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ECOPHYSIOLOGY

OF

Ruditapes decussatus

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RESUMO

Neste trabalho examinam-se as respostas fisiológicas da ameijoa *R. decussatus*, da Ria Formosa, Faro, em relação aos seguintes factores de tensão ambiental: hipoxia (PO_2 11, 6, 3 e 1,2 kPa) e anoxia; aumento agudo de temperatura (20, 27 e 32 °C), e o seu efeito na resistência à exposição ao ar (a 20, 28 e 35 °C); velocidade da corrente (0,6, 3, 8, 17, 24 e 36 cm. s^{-1}), turbidez (10, 100 e 300 mg. l^{-1}) e a eficiência de retenção de partículas de diferentes dimensões em condições de turbidez (10 e 100 mg. l^{-1}); contaminação por cobre considerando tanto a exposição aguda a elevadas concentrações (0,1-10 mg Cu. l^{-1}), como a exposição crónica a concentrações ambientais (0,01 mg Cu. l^{-1}).

As taxas de filtração, de respiração, e de excreção e a eficiência de absorção do alimento são utilizadas em termos energéticos para avaliar o comportamento em diferentes condições de tensão ambiental e na determinação da energia disponível para o crescimento ("scope for growth", SFG).

Observaram-se taxas de respiração e de filtração independentes das condições de hipoxia até 12 kPa e 6 kPa, respectivamente. As taxas de metabolismo em anoxia, medidas por dissipação de calor, representam apenas 3,6 % das taxas metabólicas em condições de normoxia. Em condições extremas de hipoxia (< 3 kPa), SFG reduz-se para 14 % dos valores em normoxia.

A taxa repiratória é independente da temperatura na gama 20 a 32 °C, mas o decréscimo da taxa de filtração conduz a valores negativos de SFG a 32 °C. A entreabertura das valvas durante exposição ao ar e o rápido metabolismo aeróbio provoca 100 % de mortalidade em 20 horas a 35 °C, em 4 dias a 28 °C e em 5 dias a 20 °C.

Baixas velocidades da corrente (≤ 8 cm. s^{-1}) permitem elevadas taxas de filtração. Pressões de fricção (≥ 0,9 Pa) induzem o movimento do sedimento e perturbam o processo de obtenção de alimento, daí resultando o decréscimo das taxas de filtração (a 36 cm. s^{-1} diminui para 10 % dos valores máximos). A capacidade desta espécie de ejectar a corrente exalante (filtrada) a um nível diferente da corrente inalante é uma importante adaptação às correntes de baixa velocidade, que ocorrem em ambientes confinados ou abrigados.

O controlo da ingestão de elevadas concentrações de seston (> 100 mg. l^{-1}) é feito por redução da taxa de filtração para 30 %, diminuindo assim a quantidade filtrada, e por rejeição de partículas através da produção de pseudofezes.

A retenção de partículas na gama 3 a 8 μm de diâmetro faz-se com elevada eficiência (70-100 %), constituíndo uma vantagem quando as microalgas de que se alimentam ocorrem diluidas por elevadas concentrações de finas partículas inorgânicas ressuspendidas pelas correntes.

R. decussatus apresenta um comportamento evasivo, por fecho das valvas, quando exposta de forma aguda a elevadas concentrações de cobre, pelo que a aplicação dos ensaios ecotoxicológicos agudos de curta duração (96h) com cobre não é possível. A exposição a concentrações ambientais de cobre não tem efeitos letais no período de exposição utilizado (20 dias), mas SFG decresce para 30 %, o que indica ocorrência e persistência de perturbações das funções fisiológicas.

Este trabalho comprova a sensibilidade da abordagem da ecofisiologia do ponto de vista energético e a integração dos efeitos perturbadores na medida de SFG, não só em relação à antecipação de efeitos irreversíveis, como por exemplo, no estudo de efeitos de contaminações ambientais, mas também para detectar alterações provocadas por factores naturais, e a capacidade de adaptação dos organismos, por regulação fisiológica, a um ambiente sempre em mudança.

ABSTRACT

The physiological responses of the clam *R. decussatus* from the Ria Formosa, southern Portugal, were examined in relation to normoxia, hypoxia (11, 6, 3 and 1.2 kPa) and anoxia; acute elevation of temperature (at 20, 27 and 32 °C), and its effect on the resistance to air exposure (at 20, 28 and 35 °C); current velocity (0.6, 3, 8 17, 24 and 36 cm. s^{-1}) and turbidity (10, 100 and 300 mg. l^{-1} dry weight of particulate matter), and the efficiency of this species in retaining particles of different size (at 10 and 100 mg. l^{-1}); and to copper contamination considering both short-term acute exposure to high levels (0.1-10 mg Cu. l^{-1}) and chronic environmental levels (0.01 mg Cu. l^{-1}).

Clearance rates, respiration rates, absorption efficiency and excretion rates were assessed through the physiological energetics in terms of the energy budget and scope for growth (SFG).

Stress independent respiration rates (R) and clearance rates (CR) were observed in relation to hypoxia down to 12 kPa and 6 kPa, respectively. Anoxic rates were 3.6 % of normoxic rates. Scope for growth was greatly reduced under extreme hypoxia (14 % of SFG in normoxia).

Respiration rate was temperature independent in the range 20-32 °C but the decline in clearance rate resulted in negative SFG at 32 °C. Gaping during air exposure and the maintenance of faster aerobic metabolism led to 100 % mortality in 20 hours at 35 °C, 4 days at 28 °C and 5 days at 20 °C.

Low current velocities ($\leq$ 8 cm. s^{-1}) supported high clearance rates. Shear stresses $\geq$ 0.9 Pa induced sediment movement and disturbed the feeding processes resulting in decreased clearance rates (at 36 cm. s^{-1}, is 10 % of maximum CR). The observed ability of jetting out depleted water at a different level than the one of the inhalant current results is an important adaptation of clams to the slow currents of sheltered environments.

Ingestion at high seston concentrations (> 100 mg. l^{-1}) is controled by reducing the amount filtered, lowering CR (to 30 % of CR at low seston loads) and producing pseudofeces.

Observed efficient retention of particles (70-100 %) in the range 3 to 8 μm is beneficial when algal cells are diluted by fine silt particles as it is likely to occur in the clams natural environment.

R. decussatus in the short term escaped the exposure to copper by valve closure and therefore acute tests are not applicable to adult clams of this species. At environmental levels chronic exposure to copper did not induce lethal effects during the exposure period (20 days), but scope for growth was reduced to *c.* 30 %, indicating sustained impairment of physiological functions.

The sensitivity of the physiological energetics and the integrated scope for growth measurement in assessing stress effects caused by natural environmental factors was highlighted.

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1. General Introduction

1. General introduction

1.1. Ecophysiology

Physiology is the study of the functions of living organisms, how they regulate these functions, and about how these functions are related and integrated into a functioning organism (Schmidt-Nielsen, 1990). Any organism utilizing the environment resources must also be able to cope with the difficulties it presents. Thus a comparative and environmental approach provides deeper insight into physiology.

Ecophysiology (or physiological ecology) is the study of how an organism is adapted to function in a particular environment. Generally we must begin with the knowledge of the natural conditions normally experienced by the organism. The tolerance limits must be appreciated, preferably as a result of observations both in the field and in laboratory experiments.

As all species, bivalves are capable of some degree of compensation for environmental change, the extent of this capacity and the flexibility of the individual's response have to be evaluated if we wish to understand the species ecology (Bayne *et al.*, 1976a). The basic pattern of the organisms response is important to assess physiological compensation and therefore the steady-state values of the relevant physiological rates functions must be known. This may require long term experiments and frequent measurement of the physiological processes but, on the other hand, short term experiments are essential in assessing physiological responses to sudden environmental changes.

Integration of all the physiological responses yields the most complete understanding of how the animal functions in its normal world. They are of great importance in ecology as they suggest the use of quantitative indices in describing and predicting the physiological condition of the whole organism through the physiological responses to environmental change (Bayne *et al.*, 1976b).

1.2. Physiological energetics and scope for growth

Growth rate is a fundamental measure of the physiological fitness / performance and has been used as a measure of environmental quality and stress effects. However, bivalve growth is often difficult to quantify and interpret specially in relation to pollution. Therefore, the determination of the energy available for growth and reproduction (scope for growth), based on the physiological analysis of the energy budget (physiological energetics) rather than direct measurement of growth itself, has proved to be particularly useful in assessing the biological effects of environmental stressors (Widdows & Donkin, 1992).

Physiological energetics and scope for growth have been widely applied to assess the response and adaptation of bivalves to natural environmental stressors. Energy aquisition and growth is directly related to food concentration, thus many studies have examined the responses of the feeding processes to different ration levels and/or food quality in the mussel *Mytilus edulis* (Widdows *et al.*, 1979, Kiørboe *et al.*, 1981, Riisgård

& Randløv, 1981, Bayne *et al.*, 1987, Riisgård, 1991), *M edulis*, *Cerastoderma edule* and *Venerupis pullastra* (Foster-Smith, 1975), *Ostrea edulis* (Grant *et al.*, 1990), *Mercenaria mercenaria* (Bricelj & Malouf, 1984), *C. edule* (Navarro *et al.*, 1994).

The sensitivity of the physiological processes and the ability to compensate for natural environmental changes have been examined regarding declining oxygen tensions in *M. edulis* (Bayne, 1971, Riisgård & Randløv, 1981, Wang & Widdows, 1991, 1993b), *Crassostrea virginica* (Widdows *et al.*, 1989), *Abra tenuis* (Wang & Widdows, 1993a); temperature in *M. edulis* (Widdows & Bayne, 1971, Widdows, 1976, 1978a); current velocity in *M. edulis* and *Placopecten magellanicus* (Wildish *et al.*, 1987, 1992), *M. mercenaria* and *C. virginica* (Grizzle et al., 1992), *Modiolus modiolus* (Lesser *et al.*, 1994); salinity in *M. edulis* (Bayne, 1975 *in* Bayne *et al.*,1985); aerial exposure in *M. edulis* and *C. edule* (Widdows & Shick, 1985, Shick *et al.*, 1986); tidal height in *Macoma balthica* (Wilson & Elkaim, 1991), as well as changes related to season and to the reproductive cycle of *C. edule* (Newell & Bayne, 1980), and M. edulis (Newell & Thompson, 1984). Recently Pérez-Camacho *et al.* (1994) and Beiras *et al.* (1994) have examined some aspects of the physiological energetics of *Ruditapes decussatus* larvae.

Physiological energetics integrate and provide insight into some of the primary mechanisms of toxicity that are both biologically and environmentally important. As reviewed by Widdows & Donkin (1991), it reflects major mechanisms of toxicity such as: non-specific narcosis and neurotoxic effects on gill cilia control affecting ciliary feeding activity (caused by exposure to hydrocarbons, dinoflagellate toxins and copper), alterations on the respiration rate due to uncoupling of oxidative phosphorylation or inhibition of oxidative metabolism (caused by exposure to TBT, DBT, phenols and hypoxia), and toxic effects on the membrane structure and function affecting digestion and absorption processes (caused by exposure to hydrocarbons).

Physiological energetics are concerned with the gains and losses of energy from the standpoint of the whole organism. All the processes that compromise the energy balance are capable of variation in response to changes in the environment and thus the balances between these processes are the substance of physiological adaptation (Bayne & Newell, 1983).

Scope for growth (SFG) is thus a general stress index and represents a non-specific response to the sum of environmental stimuli, including stressors like pollutants and natural physical and biological factors, providing measurements of the overall impact of environmental changes and complementing the more specific responses at the cellular level (Viarengo & Canesi, 1991).

The SFG index has been widely used in environmental monitoring assessment as well as in laboratory experiments to investigate the effects of different stress factors. The SFG of the mussel *M. edulis* has been used in the assessment of the effects of ration, body size, temperature and season (Widdows, 1978a), of trace metals and chlorinated compounds (Martin *et al.*, 1984), of air exposure (Widdows & Shick, 1985), of hydrocarbons (Widdows *et al.*, 1987), of diesel oil and copper (Widdows & Johnson, 1988), of copper exposure and accumulation of stress proteins (Sanders *et al.*, 1991), of tributyltin and dibutyltin (Widdows & Page 1993). The SFG index has also been applied to other bivalves, such as *C. edule* in the assessment of air exposure effects (Widdows & Shick, 1985) and of variable food quality and quantity (Navarro *et al.*, 1994), *C. virginica* larvae and hypoxia effects (Widdows *et al.*, 1989), *Ostrea edulis* in relation to different food concentrations (Beiras *et al.*, 1994), *Arca zebra* and field exposure to hydrocarbons and TBT (Widdows *et al.* 1990), *P. magellanicus* and diet effects (Grant & Cranford, 1991) and *M. modiolus* in relation to flow and seston availability (Lesser *et al.*, 1994).

The clam *R. decussatus* is grown extensively on the intertidal mudflats of the Ria Formosa, southern Portugal, with a production of 8000 tons per year, corresponding to *c.* 10 billion PTE (*c.* 70 million USD per year). Ninety percent of the production is exported (Ferreira *et al.*, 1989). *R. decussatus* is thus an economically important species and clam farming is a very important activity for the nearby populations exploiting this resource.

Being burrowing siphonate bivalves, clams may be stressed by natural factors such as hypoxia and anoxia in the sediments and at the water sediment interface; elevated temperatures due to exposure of the tidal flats during lowtide; high current velocities due to tidal currents causing resuspension of fine particulates and thus excess turbidity; and anthropogenic factors like pollution. Size, age and the physiological condition (i.e. stage of the reproductive cycle) will interact with these factors and determine the clams capacity to adapt to environmental changes.

Since 1983 mass mortality of clams in the Ria Formosa has been reported especially during summer. The reasons for this are uncertain (Ferreira *et al.,* 1989), but possibly interacting stress factors are not allowing physiological compensation thus leading to mortality.

Little work has been carried out on the ecophysiology of *R. decussatus* and nothing is known about the way this species copes with environmental changes or about the way physiological processes (from the point of view of the energy budget) allow for compensations that ensure the ecological fitness of the clams.

1.3. Objectives

In this study we will examine the physiological responses of *R. decussatus* to: environmental hypoxia and anoxia; acute elevation of temperature and its effect on the resistance to air exposure; different current velocities and different turbidity levels and the efficiency of this species in retaining particles of different size; and to copper contamination considering both acute exposure and chronic environmental levels.

The physiological responses of the clam *R. decussatus* to these stress factors will be assessed through the physiological energetics in terms of the scope for growth index and emphasis will be put in the sensitivity of the physiological approach as an early warning for deviations from normal performance.

2. GENERAL METHODOLOGY AND PRELIMINARY STUDIES

2. General methodology

This chapter deals with the general methods that are common to all or part of the experiments performed (Chapters 3, 4, 5 and 6). They are described in this section to avoid repetition.

The general methods include:

- methods used to culture algal food for the clams
- methods used for physiological measurements: clearance rates, respiration rates, excretion rates and absorption efficiency
- calculation of scope for growth (SFG).

Preliminary studies include:

- relation between physiological rates and body size,
- weight standardization of physiological rates
- level of algal cells inducing pseudofeces production
- characterization of the field area

2.1. Methods used for the algal cultures

Phaeodactylum tricornutum was used as food for the clams in all the experiments performed in Portugal, at the Departamento de Ciências e

Engenharia do Ambiente (DCEA) in Lisbon. The diatom was maintained in a semi-continuous culture.

Several solutions were prepared and mixed to make up the culture medium, the composition of which (adapted from Guillard, 1975), is given in Appendix I. Experiments with copper were to be performed therefore the culture medium was free of EDTA to avoid complexation.

In the experiments performed at Plymouth Marine Laboratory (PML), a semi-continuous culture of *Isochrysis galbana* was used to feed the clams. The preparation of the media is similar except that EDTA is present and the silicate solution is not added.

The media were autoclaved in 5 l Erlenmeyers at 121 °C for 20 minutes at 1 atm.

2.2. Methods used for the physiological measurements

The physiological measurements needed to calculate scope for growth (SGF) include: clearance rate, respiration rate, food absorption efficiency and ammonia excretion rate. For these measurements we used the general procedures described by Bayne *et al.* (1985).

Experiments on the influence of hypoxia, elevated temperature and copper accumulation on the physiological performance of clams were performed at DCEA in a temperature controlled room, set at 20 ± 1 °C, using a static approach.

Experiments on the influence of current velocity and turbidity on the clearance rate and particle size selection of the clams were performed at PML at 20 °C using a specially designed flume (for details please see Chapter 5).

All the animals were brushed carefully to completely clean the shell of fine sediment and then numbered. They were allowed to acclimate to the experimental conditions for at least 48 hours and at DCEA they were fed with a grown culture of *Phaeodactylum tricornutum* twice daily (*c.* 100000 algal cells per individual per day) and the water was changed every two days. At PML clams were placed in a flow-through system and were fed continuously with *Isochrysis galbana* at a concentration of *c.* 3000 cells per ml.

Care was taken when handling the animals, avoiding pressure on the valves and physical shock when transferring them to the experimental containers.

2.2.1. Clearance rates

Clearance rate defined as the volume of water cleared of particles per unit time, was determined indirectly by monitoring the decline in algal cells in a "closed system".

The following procedure was used in the experiments performed at DCEA. The procedure used in the flume experiments at PML, though basically the same, is detailed in Chapter 5.

Plastic beakers with 2 l of filtered (0.45 μm) seawater and with one animal each were used. These were placed on insulating plates above magnetic stirrers, to avoid a temperature increase resulting from the heat generated by the stirrer. To avoid any physical disturbance, the animals were placed on the side of a perforated platform at the bottom of the beaker, above a bar magnet rotating at a slow speed. A control beaker (without animal) was also run.

Thirty minutes was generally enough time for the animals to resume pumping, after which a known volume of algal culture was added. The initial algal concentration in the beakers was ≤ 25000 cells. ml^{-1}, in order to avoid pseudofeces formation and inhibition of the feeding processes.

After allowing 5 minutes for the algal cells to mix thoroughly, a sample of *c.* 20 ml was taken from the center of each beaker with a syringe and tubing. Samples were collected every 20 minutes for 1h 40 min. (i.e. on 6 occasions). The cell concentrations were counted immediately using an electronic particle counter Coulter Counter® Model ZM or D, fitted with a 140 µm aperture tube. Cell concentrations were the mean of 3 to 4 counts.

Clearance rate was calculated using the equation (Coughlan, 1969):

$$CR = V\,(\ln C_1 - \ln C_2) / t$$

where CR is the clearance rate, V is the volume of water used, C_1 and C_2 are the cell concentrations between two sampling times and t is the time increment.

In order to avoid considering periods when individuals may not be pumping regularly, the maximum CR is calculated over two consecutive time increments during which the decline in algal cell concentration is greatest.

Any significant changes in the clearance rates of the control, due either to cell division or settlement, were subtracted from the experimental rates. In order to relate the clearance rate to the dry tissue weight of the animals (l. h^{-1} g^{-1}), the soft parts of the clams were removed from the shell and dried for 48 h at 60 °C.

Clearance rates measurements were weight standardized to a 0.3 g dry weight (dw) animal. Ingestion rates were calculated from CR multiplied by the concentration of food (POM mg. l^{-1}).

2.2.2. Respiration rates

Rates of oxygen consumption were measured individually in closed acrylic respirometers of *c.* 800 ml. Filtered (0.45 µm) air saturated sea water, or water at a lower oxygen partial pressure (PO_2) in the hypoxia experiments, was carefully added to each respirometer ensuring that all air bubbles were eliminated.

The water was stirred slowly by means of a rotating magnet, i.e. stirrer bar, beneath a perforated plate supporting the animal. An insulating plate was placed under each respirometer to avoid any transfer of heat from magnetic stirrer.

The clams were allowed 20 to 30 minutes to resume pumping before rates of O_2 uptake were recorded. In the hypoxia experiments this period of time was sometimes extended (1-2h).

The rate of decline in oxygen within the respirometer chamber was measured with a calibrated oxygen microelectrode, Strathkelvin Instruments® Model 1302, inserted in the respirometer and connected to an oxygen meter, Strathkelvin Instruments® Model 781.

The oxygen uptake was measured 10-15 minutes after the stabilization of the microelectrode readings and when the uptake was linear. The decline in O_2 was monitored by a strip chart recorder connected to the oxygen meter.

The rate of oxygen consumption was then calculated using the following equation

$$R = [C_{to} - C_{t1})] \times V \times 60 / (t_1 - t_0)$$

where R is the rate of oxygen uptake ($\mu M\ O_2.\ h^{-1}$), C_t is the concentration of oxygen in the water ($\mu M\ O_2.\ l^{-1}$) at time t, V is the volume of the water in the respirometer and t_0 and t_1 the initial and end times (in minutes) of the measurement period.

The respiration rate is related to the dry weight of the animals ($\mu M\ O_2.\ h^{-1}\ g^{-1}$). The dry weight is obtained after oven drying the soft parts of the clams for 48 h at 60 °C.

Respiration rates measurements were weight standardized to a 0.3 g dw animal.

2.2.3. Excretion rates

The rate of ammonia excretion forms a relatively small proportion (< 5 %) of the metabolic energy expenditure (Widdows, 1993) and is therefore generally omitted from physiological energetics measurements in the calculation of scope for growth.

Nevertheless, for higher temperatures the contribution of excretion can be higher (Bayne, 1976, Bayne & Scullard, 1977) and was therefore calculated for the set of temperature experiments performed.

Ten animals were placed individually in beakers filled with 200 ml of filtered (0.45 μm) sea water and placed in a water bath held at the experimental temperature. One beaker without animal acted as the control. After 1 to 2 hours of incubation two samples were taken from each beaker and the ammonia concentrations (as NH_4-N) were determined by the phenol-hypochlorite method described by Solórzano (1969).

The ammonia excretion rates were calculated using the equation

$$U = (T - C) \times (V / 1000) / t$$

where U is the rate of ammonia excretion (μM NH_4-N. h^{-1}), T is the ammonia concentration (μM) in the sample, C is the ammonia concentration (μM) in the control, V is the volume (ml) of sea water in which the animal is incubated and t is the incubation time (h).

The excretion rate is related to the dry weight of the animals (μM O_2. h^{-1} g^{-1}). The dry weight is obtained after oven drying the soft parts of the clams for 48 h at 60 °C.

Excretion rates measurements were weight standardized to a 0.3 g dw animal.

2.2.4. Absorption efficiency

The absorption efficiency (AE) was measured using the ratio method of Conover (1966), which represents the efficiency with which organic material is absorbed from the ingested food. It assumes that an animal can digest and absorb the organic component of the food but not the inorganic fraction.

The Conover method is based on the ratio between the organic content of food and feces and is calculated from the equation:

$$AE = (F - E) \times 100 / [(1 - E)F]$$

where AE is the absorption efficiency (%), F is the organic content of the food ingested and E is the organic content of the feces.

To determine the organic content the ash-free dry weight : dry weight ratio of both food and feces was calculated according to the following procedures:

The algal culture (30 ml) of known cell concentration was filtered through, washed, ashed and pre-weighed 4.5 cm glass fiber filters (Whatman® GF/C). Salts were then washed out with 0.5 M ammonium formate (10 ml). Feces accumulated in the beakers from the clearance rates measurements were collected by pipetting onto washed, ashed and pre-weighed GF/C filters. The salts were washed out with 0.5 M ammonium formate (10ml). Generally feces from 2 to 3 individuals were pooled in order to have a measurable quantity.

Both food (algal cells) and feces filters were obtained in triplicate. Blank GF/C filters, also washed, ashed and pre-weighed were used with each batch of filters for corrections of weight change with daily variations of humidity.

The filters were oven dried at 105 °C for 48 hours and weighed, and were then ashed in a muffle furnace at 450 °C for 4 hours and weighed again to calculate the weight of organic material combusted. Filters were cooled in desiccators before weighing.

2.3. Calculation of scope for growth (SFG)

As the energy budget of an animal represents an integration of the basic physiological responses, such as feeding, food absorption, respiration, excretion and production, each of these physiological responses can be converted, by appropriate conversion factors, into energy equivalents ($J. h^{-1}$). Food energy value was calculated using the energy equivalent 1 mg POM = 23 J. (Widdows *et al.* 1979), energy loss through respiration was calculated using the energy equivalent 1 µmol O_2 = 0.456 J (Gnaiger, 1983), energy loss through excretion was calculated using the energy equivalent 1 µmol $N\text{-}NH_4$ = 0.349 J (Elliot & Davidson, 1975).

The scope for growth (SFG), is quantified by means of the balanced energy equation of Winberg (1960):

$$C = P + R + E + F$$

where C is the total consumption of food energy, P is the total production of shell, somatic tissue and gametes, R is the respiratory energy expenditure (i.e. the costs of maintenance, feeding, digestion and growth), E is the energy lost in excreta, and F is the fecal energy loss. The absorbed ration is the product of the energy consumed (C) and the efficiency of absorption of energy from the food. Production may then be expressed as:

$$P = A - (R+E)$$

where P (SFG) is estimated from the difference between the energy gains, energy absorbed from food (A), i.e., ingestion rate x AE, and the energy losses, energy expenditure via respiration (R) and excretion (E). Scope for growth can range from maximum positive values under optimal conditions, declining to negative values when the animal is severely stressed and utilizing its body reserves (Widdows, 1993).

2.4. Physiological rates and body size

In order to introduce a weight correction into comparisons between animals of different sizes, the relationship between body size and metabolic rates was determined. It is well known that metabolism is proportional to a constant power of body weight as described by the allometric equation

$$Y = a\,X^{b}$$

where Y is the physiological rate (as for instance oxygen consumption or clearance rate), X is the body size (in weight), b is the exponent and a denotes the level of the metabolic rate of an organism of unit body weight.

The value of *a* varies widely according to a variety of factors, activity and temperature being the most important (Bayne & Newell, 1983). The weight exponent *b* is less variable and when data from different bivalves are pooled it approaches a value between 0.66 to 0.82 (Winter, 1978) and 0.75 (Jørgensen, 1976) for clearance rates, and 0.67 and 0.75 for oxygen consumption (Zeuthen, 1953 and Hemmingsen, 1960 *in* Bayne & Newell, 1983). For clearance rates *b* values are generally lower than for respiration rates indicating an increase in the metabolic costs of feeding in larger individuals (Bayne & Newell, 1983).

2.4.1. Clearance rates

The relationship between body size and filtration rate was determined in March when small individuals are introduced in the clam farm as “seed”. The clearance rates of 23 individuals representing sizes between 17 and 35.7 mm and corresponding to 34.9 to 387.3 mg dw were measured. The fitted equation gave a *b* value of 0.65.

The relation between body size and clearance rate for a variety of infaunal bivalves and *Mytilus edulis* is summarized in Table 2.1, and the value found in this work agrees well with values from different authors.

2.4.2. Respiration rates

The relation between oxygen consumption and body size was determined in June, September and November 1993, using 8, 23 and 18 individuals respectively. In June, individual size ranged between 27.0 and 40.1 mm corresponding to 0.21 to 0.72 g dw, in September individual size ranged

Table 2.1. Values for the slopes (b) of regression lines relating measures of clearance rates to body weight by means of the allometric equation for some suspension-feeders bivalves (based on Bayne & Newell, 1983).

Organism	*b*	Reference
Mytilus edulis	0.56	revised by Winter (1978)
Mytilus edulis	0.72	Riisgård & Møhlenberg (1979)
Mytilus californianus	0.46	revised by Winter (1978)
Mytilus chilensis	0.59 - 0.62	Navarro & Winter (1982)
Pecten irradians	0.82	revised by Winter (1978)
Arctica islandica	0.66	revised by Winter (1978)
Modiolus modiolus	0.74	revised by Winter (1978)
Cerastoderma edule	0.58	revised by Winter (1978)
Cerastoderma edule	0.56	Newell & Bayne (1980)
Cerastoderma lamarcki	0.63	revised by Winter (1978)
Mercenaria mercenaria	0.31	Hibbert (1977)
Mercenaria mercenaria	0.73	revised by Winter (1978)
Argopecten irradians	0.58	Kirby-Smith (1972)
Ostrea edulis	0.84	Rodhouse (1978)
Chlamys islandica	0.60	Vahl (unpublished)
Venerupis corrugatus	0.62	Stenton-Dozey & Brown (1994)
Ruditapes decussatus (larvae)	0.37 - 0.94	Pérez-Camacho *et al.* (1994)
Ruditapes decussatus	0.65	Sobral (this study)

between 25.9 to 40.3 mm, dry weight 0.13 to 0.43 g, and in November size ranged from 24.7 to 43.9 mm and dry weight from 0.15 to 0.68 g.

The influence of the reproductive cycle on the dry weight is particularly noticeable in June, when dry weights are higher at the beginning of the spawning season, declining to low weights in the immediate post spawning period.

This condition is reflected in the variability of the *b* values found on the three occasions, 0.73, 0.42 and 0.99 respectively.

In fact the scatter of the data about the regression lines in most experimental studies makes the differences in slopes statistically insignificant and for this reason a common mean weight exponent is calculated from all the experimental data (Bayne & Newell, 1983).

The significance of the differences between the fitted parameters of the regression lines obtained from the three experiments was determined by an analysis of covariance (Sokal & Rolf, 1969). Regressions were found not to be significantly different at P = 0.01 and so a common weight exponent was calculated.

The value of the weight exponent, 0.71, is similar to the generally accepted power coefficient of body mass (0.75), when aerobic energy expenditure is measured as oxygen uptake (Bayne *et al.*, 1976; Bayne & Newell, 1983, Widdows, 1987). This indicates that metabolism is limited by the surface area available for oxygen diffusion, declining on a weight-specific basis with increasing size (Hawkins & Bayne, 1992). Our results fit well with the values generally found for the weight exponent of different bivalves as shown in Table 2.2.

Table 2.2. Values for the slopes (*b*) of regression lines relating measures of oxygen consumption rates to body weight by means of the allometric equation for different bivalves (based on Bayne & Newell, 1983).

Organism	*b*	Reference
Mytilus edulis	0.67 - 0.74	Bayne *et al*. (1973)
Mytilus edulis	0.87	Famme (1980)
Mytilus californianus	0.65	Bayne *et al*. (1976)
Ostrea edulis	0.66	Newell *et al*. (1977)
Ostrea edulis	0.75 - 1.09	Rodhouse (1978)
Crassostrea virginica	0.60 - 0.73	Dame (1972)
Scrobicularia plana	0.75	Hughes (1970)
Cerastoderma edule	0.44	Boyden (1972a, b)
Mulinia lateralis	0.34 - 0.88	Shumway (1983)
Glycemeris glycemeris	0.45	Brand & Morris (1884)
Ruditapes philippinarum	1.06	Bodoy, *et al.* (1986)
Ruditapes decussatus	0.87	Bodoy *et al*. (1986)
Ruditapes decussatus	0.42 - 0.99	Sobral (this study)

2.5. Weight standardization of physiological rates

As we have seen the value of the weight exponent *b* varies mainly with the size range studied and temperature, the time of the year as related to the reproductive cycle, the ration, the activity level and thus the general physiological condition of the animals.

Nevertheless, according to Bayne & Newell (1983), a common mean weight exponent of 0.70 is generally applicable. As our values do not differ significantly from 0.70, this value was used to weight standardize clearance and respiration rates .

To standardize physiologic rates to a certain weight the following formula was used (Bayne *et al.*, 1987)

$$Y_s = (W_s / W_e)^b \times Y_e$$

where Y_s is the physiological rate of the standard sized animal, W_s is its weight, W_e is the measured weight of the experimental animal, Y_e is the uncorrected physiological rate and b is the corresponding weight exponent.

2.6. Pseudofeces production

Pseudofeces production is a mechanism by which bivalves reject excess particles being pumped into the mantle cavity. Special ciliary tracts (rejection tracts) convey these excess particles embedded in strings of mucus out of the inhalant siphon. The seston concentration that induces pseudofeces formation varies with the species and probably reflects an adaptation to the environmental seston variation as shown by Widdows *et* *al*. (1979), for the mussel *M. edulis* and Foster-Smith (1975), for *M. edulis*, *Cerastoderma edule* and *Venerupis pullastra*.

When calculating scope for growth, measuring ingestion from clearance rates, i.e. assuming that all the food filtered from the water is ingested, it is necessary to keep food levels below the threshold of pseudofeces production.

To determine the algal concentration that induces the production of pseudofeces three clams was placed individually in *c.* 500 ml of filtered (0.45μm) sea water and allowed 2 hours of settlement in the experimental containers. Algal cells (*Pheaodactylum tricornutum)* were then added every 5 minutes to obtain an increasing cell concentration. The water was stirred slowly by means of a rotating bar magnet beneath a perforated plate upon which the animal was placed.

Cell numbers (mean of 3 or 4 counts) were monitored with a Coulter Counter Model ZM, fitted with a 140 μm aperture tube. The cell concentration range investigated was *c.* 7000 to *c.* 230000 cells. ml^{-1}, corresponding to a seston concentration of 0.3 to 10.0 mg. l^{-1}.

No pseudofeces were observed up to *c.* 60000 cells. ml^{-1} (*c.* 2.5 mg. l^{-1}). Direct observation of the clams showed that both siphons were still wide open up to *c.* 80000 - 90000 cells. ml^{-1} (<4 mg. l^{-1}) though some pseudofeces (thin strings of mucus) could be seen on the bottom of the containers. Above 100000 cells. ml^{-1} (>4.5 mg. l^{-1}) the marginal tentacles of the inhalant siphon were folded inwards indicating lower clearance rate, and there were mucous aggregations on the mantle edge. Apart some episodic tip constriction of the siphons, no contractions or jetting out through the inhalant siphon were observed.

2.7. Characterization of the field site

2.7.1. Introduction

All the clams (*Ruditapes decussatus* L.) used in this work came from a clam farm located near the city of Faro, in the Ria Formosa, along the south coast of Portugal.

Ria Formosa is a complex system of salt marshes, mudflats, channels and shallow lagoons, sheltered from the Atlantic Ocean by a series of barrier islands and peninsulas (Figure 2.1).

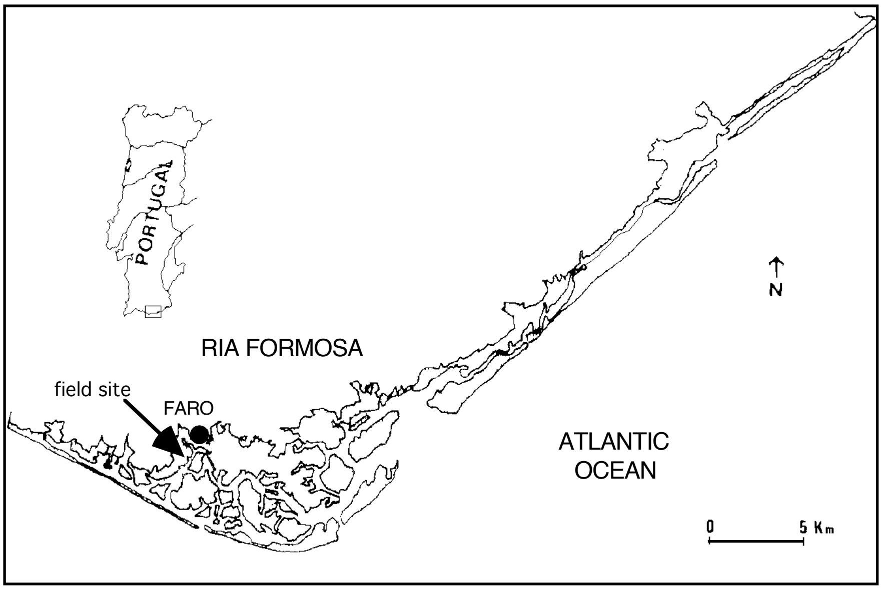

Fig. 2.1. Map of the Ria Formosa indicating the study site (→).

This system has an area of *c.* 150 Km^2, its maximum width being south of Faro and Olhão. There is no relevant freshwater input. The water exchange during the tidal cycles is high (50 to 75%), because of the average water depth being less than 3.5 m (Wolfrath, 1992). Large areas of the lagoon are covered by macrophytes.

The exposed intertidal area may reach 50 Km^2 at low water during spring tides. *R decussatus* is grown extensively on these intertidal mudflats where 1500 clam farms occupying 1000 ha are estimated to have a production of 8000 tons per year (Ferreira *et al.*, 1989), corresponding to *c.* 10 billion PTE (or 70 million USD) per year . Ninety percent of the production is exported. Clam farming is thus a very important economical activity for the nearby populations exploiting this resource.

2.7.1.1. Distribution and taxonomic position of *Ruditapes decussatus.*

R. decussatus is a burrowing siphonate bivalve largely distributed on the Atlantic coast, from England to Mauritanea and is also found in the Mediterranean Sea, being cultivated in the coastal lagoons near the Gulf of Lyon (Guellorget *et al.*, 1980) and in the Venice Lagoon (Breber, 1985).

Natural populations of *R. decussatus* can be found in sandy and muddy-sand sediments in bays, estuaries coastal lagoons and other sheltered environments, living on tidal flats and below tidal marks. They are filter-feeders pumping water through the siphons at sediment level, and they can bury in the sediment to a depth of 10 - 12 cm (Vilela, 1950). According to this author they feed primarly on planktonic and benthic algae especially diatoms.

This species was first described as *Venus decussata* (L.) and through the years it has been known by other names (synonimous) of which the most common are *Venerupis decussata* and *Tapes decussatus*. After the revision of the Family VENERIDAE by Fisher-Piette and Metivier (1971), its systematic position is the following:

Phylum MOLLUSCA
Class BIVALVIA (Linnaeus, 1758)
Order EULAMELLIBRANCHIA (Woodward, 1892)
Super-Family VENEROIDEA (Rafinesque, 1815)
Family VENERIDAE(Rafinesque, 1815)
Subfamily TAPETINAE (Fischer, 1887)
Genus *Ruditapes* (Chiamenti, 1900)
Species *decussatus* (Linnaeus, 1758)

The official denomination is therefore *Ruditapes decussatus* (L.). In portuguese it is known as “ameijoa-boa” or “ameijoa-verdadeira” and in english as carpet-shell clam of butterfish clam.

2.7.1.2. Field data

A program was designed to sample the field site on a seasonal basis for standard parameters of the water (temperature, salinity, pH, dissolved oxygen, particulate matter, PM, and particulate organic matter, POM) and sediment (temperature, pH, Eh and organic matter and pore water PM and POM) and for determination of body condition index of clams.

Information on the physical and chemical conditions of the field site, together with data on the seasonal variation of body condition of clams, was useful not only to approach realistic conditions in our experiments, like food levels and turbidity, but also to have a baseline to pursue further studies in the near future.

2.7.2. Material and methods

2.7.2.1. Sampling

Sampling took place between March 1992 and December 1993 at low tide, when most of the tidal flat was accessible. Water samples were collected from the overlying water of a shallow area of the clam farm. Pore water was collected in recently exposed areas from holes left from the sediment sampling and sieved through a 200 μm mesh. Only the first 10 cm of the muddy-sand sediment were sampled using an inverted plastic box.

Water temperature and dissolved oxygen were measured *in situ* with an oxygen probe, Consort® Model Z800, and pH with an Orion® Research Inc. electrode. In the sediment, temperature, pH and Eh were taken at 1, 3 and 5 cm depth using Orion® Research Inc. electrodes.

Clams were collected from February 1992 to December 1993 on a monthly basis whenever possible.

After collection all samples were kept in coolers at about 10-15 °C, during transportation to the laboratory. Clams were transported in a similar cooler in plastic net bags, without water.

2.7.2.2. Laboratory procedures

Salinity was measured in the laboratory using a direct titration method (Brehaut, 1982). Particulate matter (PM) and particulate organic matter (POM) in the overlying and pore waters were determined by collecting a known volume on glass fiber GF/C Whatman® filters, oven drying the filters at 105 °C for 48 hours and ashing in a muffle furnace at 450 °C for 4 hours. Filters were pre-washed with distilled water, ashed in the same manner and pre-weighed. After filtration 0.5 M ammonium formate was used to wash out salts from the filters. Organic matter in the sediment was determined in a homogenized sample by ashing in a similar way. Desiccators were used for complete cooling of samples prior to all weighing procedures.

Clams were measured (anterior-posterior length) with calipers, separated in four length classes between 25 mm and 45 mm, frozen for a few hours and allowed to open. The soft parts were washed with 0.5 M ammonium formate to eliminate salt, separated from the shell and oven dried at 60 °C for 48h to obtain dry weight (dw). Body condition was determined as the dw (mg) : length (mm) ratio (Walne, 1976), and averaged over 6 to 10 individuals .

2.7.3. Results and Discussion.

2.7.3.1. Overlying water, pore water and sediment data

Tables 2.3 and 2.4 summarize the average, minimum and maximum values found for parameters of the overlying water, pore water and sediment. Figs. 2.2 to 2.7 show the seasonal evolution of those parameters.

Table 2.3. Overlying water parameters at Faro sampling site, between March 1992 and December 1993 (average ± SE).

	S ‰	T °C	pH	DO $mg.\ l^{-1}$	PM $mg.\ l^{-1}$	POM $mg.\ l^{-1}$
1992-1993	36.3 ± 0.6	17.5 ± 1.3	7.5 ± 0.1	7.4 ± 0.6	144.3 ± 0.1	12.4 ± 3.8
highest value	37.7	24.0	7.9	9.9	471.0	32.6
lowest value	34.0	13.0	7.1	4.9	45.8	1.7

As it can be seen both by the values in the Tables 2.3 and 2.4 and the seasonal evolution of the parameters in the Figure 2.2, salinity, temperature pH and dissolved oxygen in the overlying water are within the expected values for coastal systems of this type.

Salinity is higher than normal oceanic values, temperatures are high in summer and low in winter, the sediment presenting higher thermal variation at low tide than the overlying water throughout the two years. Dissolved oxygen was always above saturation level, except on one occasion, July 1992, when the highest temperatures were registered.

Table 2.4. Pore water and sediment parameters at Faro sampling site, between March 1992 and December 1993 (average ± SE). PM-particulate matter, POM – particulate organic matter, T – temperature, Eh – redox potential.

	PORE WATER		SEDIMENT			
	PM g l^{-1}	POM g. l^{-1}	T °C	pH	Eh mV	Org. matter %
1992/1993	3.2 ± 1.1	0.3 ± 0.1	19.8 ± 1.7	7.3 ± 0.1	-65.4 ± 21.6	2.2 ± 0.3
highest value	7.4	0.9	27.0	7.54	2.9	3.6
lowest value	0.42	0.1	13.0	6.92	-140.6	1.2

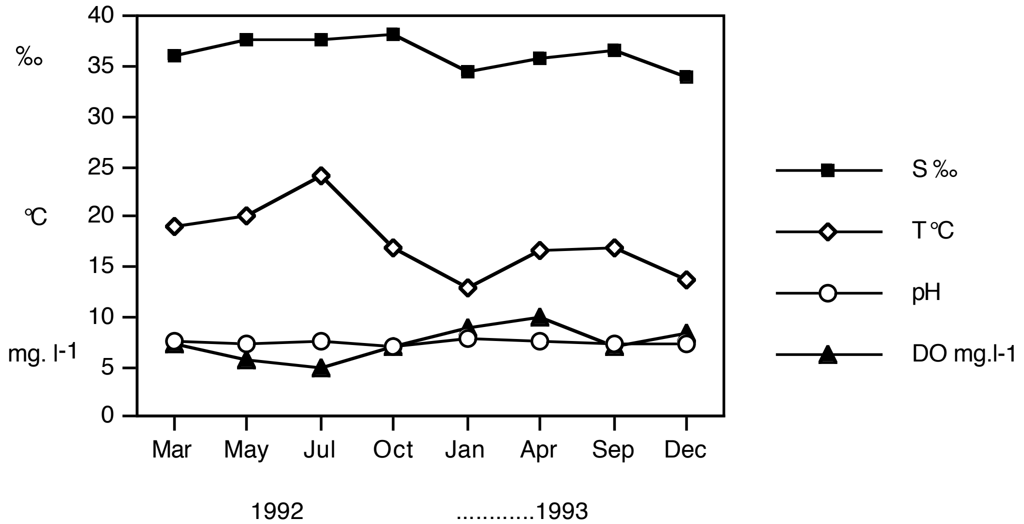

Fig. 2.2. Seasonal evolution of salinity (‰), temperature (°C), pH and dissolved oxygen (mg. l^{-1}) of the overlying water at Faro sampling site.

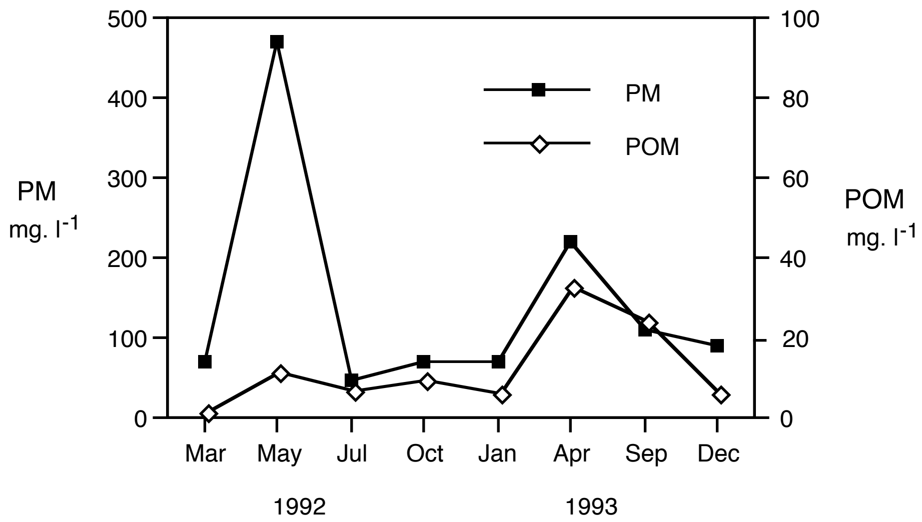

Fig. 2.3. Seasonal evolution of particulate matter (PM) and particulate organic matter (POM) in mg. l^{-1} in the overlying water at Faro sampling site.

PM and POM in the overlying water show seasonal variation (Figure 2.3), with a consistent peak in spring in both years. Most PM is inorganic and is probably associated with currents leading to resuspension of fine mud from the mud flats .

In the pore water PM is higher in spring in the first year and in autumn in the second year (Figure 2.4). POM follows roughly the same variation. Again most of the PM of pore water is inorganic, due to heavy seston load, but the amount of POM is proportionally higher in pore water than in the overlying water. This is probably related to bacteria and to microphytobenthos, macrophyte detritus and other organic debris (all under 200 µm) that become adsorbed to inorganic particles in the pore water.

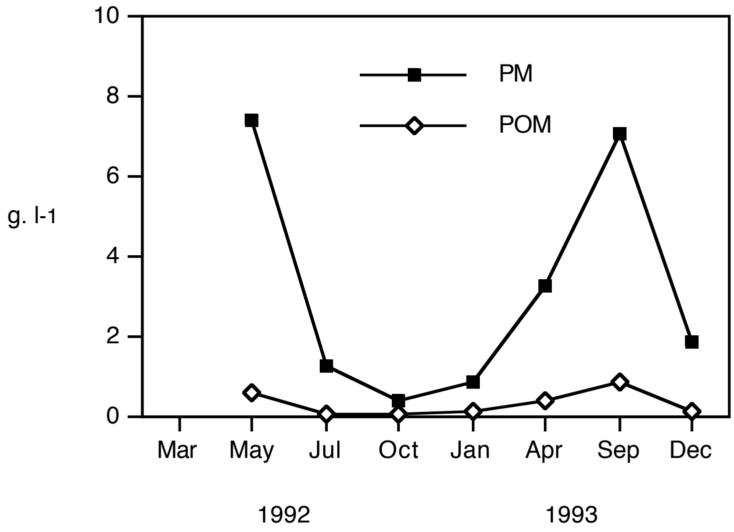

Fig. 2.4. Seasonal evolution of particulate matter (PM) and particulate organic matter (POM) in g. l^{-1} in the pore water at Faro sampling site.

Differences in temperature and pH between the three levels measured in the sediment were never higher than one degree for temperature and 0.1 for pH, the first cm showing generally higher values (Figures 2.5 and 2.6).

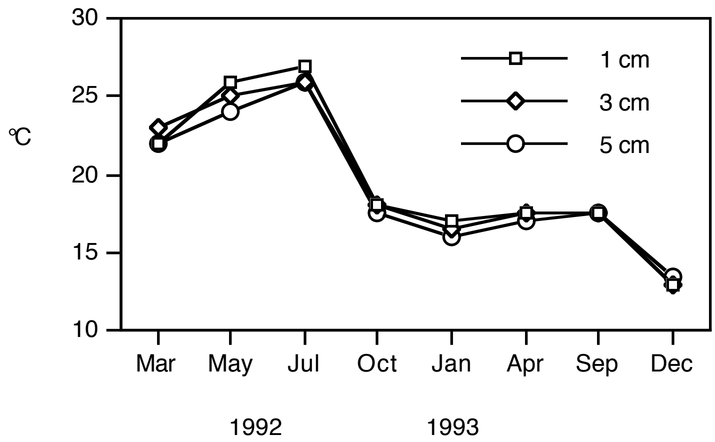

Fig. 2.5. Seasonal evolution of temperature (°C) at three levels in the sediment at Faro sampling site.

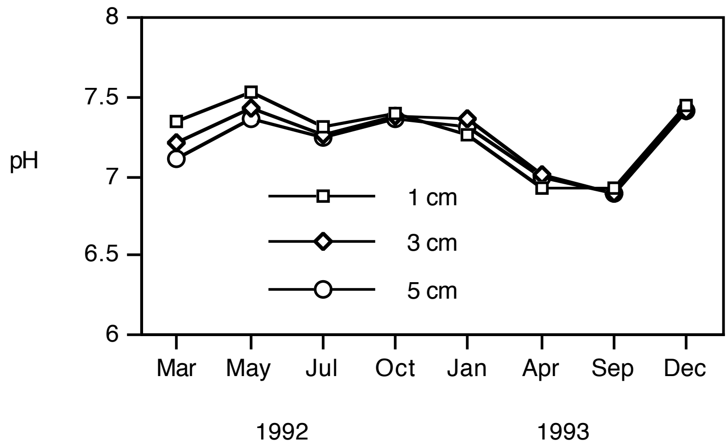

Fig. 2.6. Seasonal evolution of pH at three levels in the sediment at Faro sampling site.

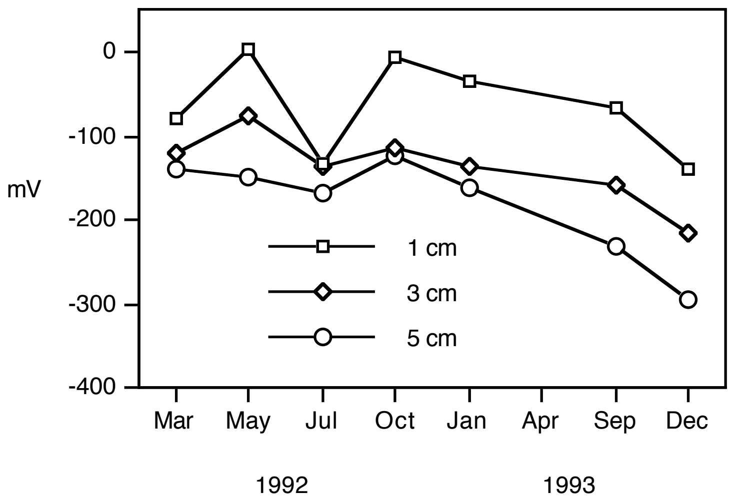

Fig. 2.7. Seasonal evolution of redox potential Eh (mV) at three levels in the sediment at Faro sampling site.

The redox potential is almost always negative in the first level and always negative at 3 and 5 cm depth (Figure 2.7), indicating that reducing conditions exist since the first cm. The black color of the sediment, indicates that reducing conditions are present since the first few mm, which is in agreement with the oxygen profiles obtained by Brotas *et al.* (1990).

2.7.3.2. Body condition index of clams

The body condition index (BCI) of *R. decussatus* varies with season due to changes in the particulate food in both the overlying water and pore water (Figures 2.3 and 2.4) and the clams reproductive cycle. In both years, despite different amplitudes, the highest index was found in June (Figure 2.8) just as clams start spawning. This was followed by a marked reduction specially in the second year.

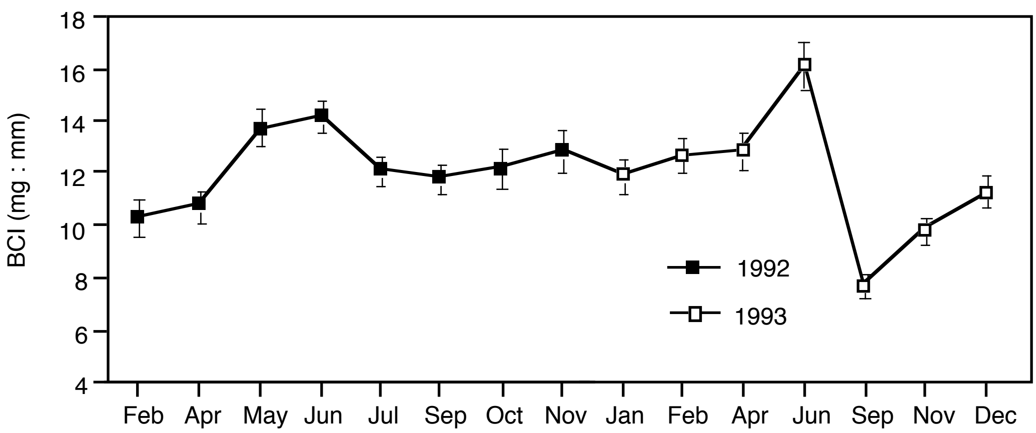

Fig. 2.8. Evolution of body condition index (BCI ± SE) of *R. decussatus* throughout the sampling period.

In summer body condition is therefore low indicating a small tissue mass for a given shell length. During autumn and winter as they start building up their body reserves the condition index slowly rises to reach their peak in late spring.

Beninger & Lucas (1984) have found the same pattern of condition variation for *R. decussatus* from Britanny and similar seasonal variations in body weight have been described for other bivalve species in the Dutch Wadden Sea by Zwarts (1991) and in Cornwall, by Newell & Bayne (1980). Work with *R. decussatus* in the Ria Formosa by Pacheco *et al.* (1988) confirm the existence of one spawning period begining in May-June and extending through Summer, thus agreeing with our findings.

3. THE INFLUENCE OF HYPOXIA AND ANOXIA ON THE PHYSIOLOGICAL RESPONSES OF *Ruditapes decussatus*.

3. The influence of hypoxia and anoxia on the physiological responses of the clam *Ruditapes decussatus*.

3.1. Introduction

Many infaunal marine bivalves live in areas where they have to cope with wide variations of dissolved oxygen concentration. It is known that in confined shallow waters like those of the Ria Formosa, the oxygen concentration can decline to low levels (c. 60 % of air saturation) at the water-sediment interface and oxygen penetration depth in muddy sand is 0.5 - 2 mm (Brotas *et al.*, 1990). These conditions are further aggravated in summer when temperatures and evaporation are higher.

Bivalves are known to tolerate extended periods of hypoxia and anoxia. Several studies (Bayne, 1971a, b, Taylor & Brand, 1975, Famme, 1980, Famme *et al.*, 1981, Riisgård & Randløv, 1981, Wang & Widdows, 1991) have emphasized that the rate of oxygen consumption as a metabolic response to declining oxygen tensions is influenced by experimental conditions, such as duration of exposure, and biotic factors, such as body size, food availability and the seasonal reproductive cycle.

Hypoxia may be induced by shell valve closure and/or depletion of oxygen in the surrounding water and physiological responses vary according to the species. Physiological compensatory mechanisms induced by environmental hypoxia may include an increase in water pumping/ventilation and/or blood circulation (Herreid, 1980), making more oxygen available to the gills and improving its distribution to the tissues which will help maintain the rate of oxygen consumption. Environmental hypoxia may induce a suppression of aerobic metabolism and an increase in anaerobic metabolism. Such suppression of aerobic metabolism serves to conserve energy and enables bivalves to survive hypoxia and anoxia for a limited period of time (Shick *et al.*, 1986, Widdows *et al.*, 1989, De Zwaan *et al.*, 1991, Wang & Widdows, 1993a, b).

Respirometric measurements are used as an indirect measure of metabolic rate but are limited to the quantification of aerobic energy metabolism. In order to provide a direct measure of anaerobic metabolism under anoxic conditions, microcalorimetric techniques have been used in this study. Calorimetry is a non-specific technique for the direct measurement of metabolic activity, detecting enthalpy changes of all reactions and so determining the rate of heat dissipation of biological processes (Widdows, 1987). This method has been particularly useful in physiological energetics studies, especially in those dealing with environmentally induced transitions between metabolic states, such as normoxic and anoxic metabolism. These studies include behavior associated with gas exchange (Shick, *et al.* 1988) and energy balance studies involving open-flow calorespirometry and biochemical measurements (Widdows 1989, Gnaiger *et al.* 1989).

The experiments outlined in this chapter are concerned with the responses of *Ruditapes decussatus* to environmental hypoxia and anoxia. Physiological responses examined were: clearance/ingestion rates and metabolic rates, quantified as total heat dissipation and oxygen consumption, and the energy balance assessed in terms of scope for growth.

3.2. Material and methods

3.2.1. Normoxia and Hypoxia

Clams were collected from the sampling site in Ria Formosa, at low tide in December 1993 and April 1994 and allowed to acclimate to laboratory conditions (15 °C and 35.6 ‰) at DCEA in Lisbon.

Experiments were performed between December 1993 and February 1994, and in May 1994 for measurements under extreme hypoxia (< 2 kPa). Clams were allowed to acclimate to the experimental temperature (20 ± 1 °C) for 2 days in a controlled temperature room. All the animals were brushed carefully to completely clean the shell of fine sediment and then numbered. Mean length was 35.2 ± 0.2 mm (SE, n = 44). They were fed with a culture of *Phaeodactylum tricornutum,* twice daily and the water was changed every two days.

Preliminary studies found that there was no significant difference between the feeding/pumping rate of clams in or out of the sediment. Therefore all experiments were performed without sediment using eight to sixteen individuals. Care was taken when handling the animals, avoiding pressure on the valves and physical shock when transferring them to the experimental containers.

Hypoxic conditions were obtained by bubbling sea water with appropriate mixtures of N_2 and O_2 gas. Generally it took about 1 hour to reach the required oxygen concentrations. During the clearance rates measurements, beakers containing the hypoxic water were covered to minimize exchanges with the atmosphere.

3.2.1.1. Physiological measurements

Scope for growth index was calculated to assess the extent to which hypoxia alters the physiological energetics of clams.

Clearance rate, respiration rate, food absorption efficiency measurements were performed according to the methods described in Chapter 2. Ammonia excretion was not quantified as this is generally a negligible fraction of the energy budget (Widdows, 1993).

All measurements were weight standardized to a 0.3 g dw animal. Ingestion rates were calculated from clearance rates (CR) multiplied by concentration of food (POM mg. l^{-1}), using the conversion factor 10^6 cells of *P. tricornutum* = 43.8 µg dw (Wang & Widdows, 1993b). Clearance rates and respiration rates were not measured on the same individuals.

Energy equivalents used to convert rates of oxygen uptake and clearance rates of algal food to joules were 1 µmol O_2 = 0.456 J (Gnaiger, 1983) and 1 mg POM = 23 J (Widdows *et al.* 1979).

All statistical analyses were performed by one-way ANOVA.

3.2.2. Anoxia

Anoxia experiments were performed with clams collected in June 1994. These were acclimatized to laboratory conditions (temperature 20 °C, salinity 34 ‰) in a flow-through system at Plymouth Marine Laboratory (PML) and were continuously fed with a culture of *Isochrysis galbana* to give an approximate concentration of 3000 cells. ml^{-1}. Clams were acclimatized for one week prior to the measurements. Due to the size of the perfusion chamber in the microcalorimeter all clams had to measure less than 2.5 cm (shell length). The anoxic rates of heat dissipation of nine individuals (mean length 24.3 ± 0.1 mm) were measured.

Individual clams were placed in a 25 ml stainless-steel perfusion chamber held within a microcalorimeter (LKB 2277 Bioactivity Monitor). Anoxia was achieved by flushing the chamber with oxygen-free nitrogen gas for 20 minutes. The calorimetric system reached a steady state after 3 hours and the rate of heat dissipation was continuously monitored for 24 hours. Another chamber without an animal acted as a reference system monitoring the baseline of heat flow.

Weight specific rates of heat dissipation are expressed as J. h^{-1} per standard 0.3 g dw animal.

3.3. Results

Fully anoxic conditions were only achieved in the calorimetric experiment, in all the other experiments the lowest hypoxia levels obtained were 0.9 kPa in respiration rates measurements and 1.2 kPa for the clearance rates measurements.

Hypoxic conditions representing 50 % and 25 % of the fully air saturated levels were usually achieved after about 1 hour of bubbling with appropriate N_2 and O_2 gas mixtures. At lower levels of hypoxia, generally 2 hours were needed to bring down the PO_2 values. At these lower levels, it was difficult to maintain a constant level of hypoxia, especially in the covered beakers where clearance rates were measured, since it was impossible to avoid mixing with air. Therefore the values of oxygen tension are an average of the overall hypoxic conditions registered in the beakers. As respiration measurements were not all started at exactly the same oxygen tension, values for the different hypoxia levels were averaged from the oxygen concentrations in the respiration chambers at the beginning of the measuring period.

3.3.1. Behavioural observations

Under normoxia 20 to 30 minutes was generally sufficient to allow all the animals to assume a "normal posture", turgid with valves open, mantle edge protruding and completely closing the paleal chamber, siphons half distended with round wide apertures, the exhalant being somewhat smaller than the inhalant and for pumping to be resumed. As oxygen tension was reduced their behavior became more variable and 1 hour was needed to resume pumping.

In some cases valves were only slightly open, there were openings in the paleal chamber and the siphons were barely visible, level with the shell edge, and not open wide. Under extreme hypoxia animals would not open, or would alternate between open and closed, siphons would be compressed and their aperture greatly reduced, and therefore respiration measurements were very variable and difficult to record.

This pattern of behavior resulted in lower clearance rates and respiration rates with increasing hypoxia. Lower clearance rates accounted for lower ingestion rates and resulted in a reduction in feces production. At the lowest hypoxic levels feces from all the animals had to be pooled to obtain a measurable sample for absorption efficiency.

Despite this general pattern of behavior, there were individuals which were apparently open and pumping efficiently but showed reduced respiration rates. Therefore any reduction in respiration was not simply due to valve closure.

3.3.2. Clearance and ingestion rates under normoxia and hypoxia

A slight increase in clearance rate was observed at 11 kPa (Figure 3.1) but until 6 kPa differences between these clearance rates and normoxia clearance rate are not significant. A statistically significant decline ($P < 0.001$, one-way ANOVA) was observed at hypoxia levels below 6 kPa. At 1.2 kPa, extreme hypoxia, the clearance rates were again statistically different ($P < 0.001$, one-way ANOVA) and corresponded to 12 % of normoxic values.

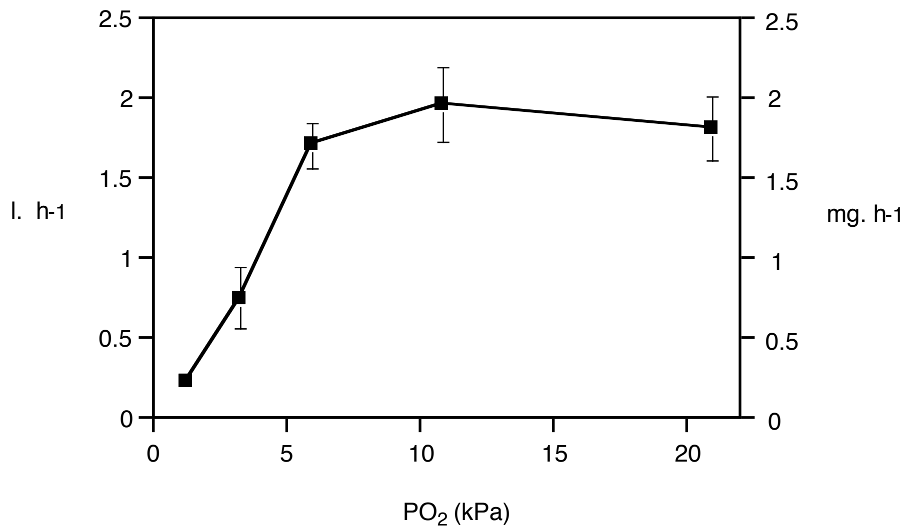

Fig. 3.1 - Clearance rates ($l. h^{-1} \pm SE$) and ingestion rates ($mg. l^{-1} \pm SE$) of *Ruditapes decussatus* under normoxia and different hypoxia levels.

The ingestion rates follow the same pattern as the clearance rates and show that feeding activity is maintained under moderate hypoxia, but below PO_2 6 kPa there is a marked decline ($P < 0.001$, one-way ANOVA).

3.3.3. Metabolic rates under normoxia, hypoxia and anoxia

Aerobic metabolic rates measured as respiration rates, decline with lower oxygen tensions but this effect is more marked below PO_2 11.7 kPa (Figure 3.2). The critical partial oxygen pressure, defined as the PO_2 level at which the metabolic rate becomes oxygen dependent, is therefore relatively high (*c.* 12 kPa). At 7.9 kPa the metabolic rate is reduced to 50 % of its normoxic value ($P < 0.01$). At the lower levels of hypoxia (7.3 to 0.9 kPa), respiration rates are held relatively constant and correspond to *c.* 35 % of normoxic rates with some evidence of regulation. In this range results were not significantly different as tested by one-way ANOVA. Anoxic rates, measured as heat dissipation, are 3.6 % of the normoxic metabolic rate.

The metabolic curve can be divided in two parts, one between 21 and 11 kPa where respiration rates are largely maintained independent of declining oxygen, followed by a marked reduction in respiration and then a second part corresponding to the extreme hypoxia range (7 to 1 kPa) where respiration rates are regulated (i.e. maintained independent of declining PO_2) albeit at a lower level (36 % of normoxic rate).

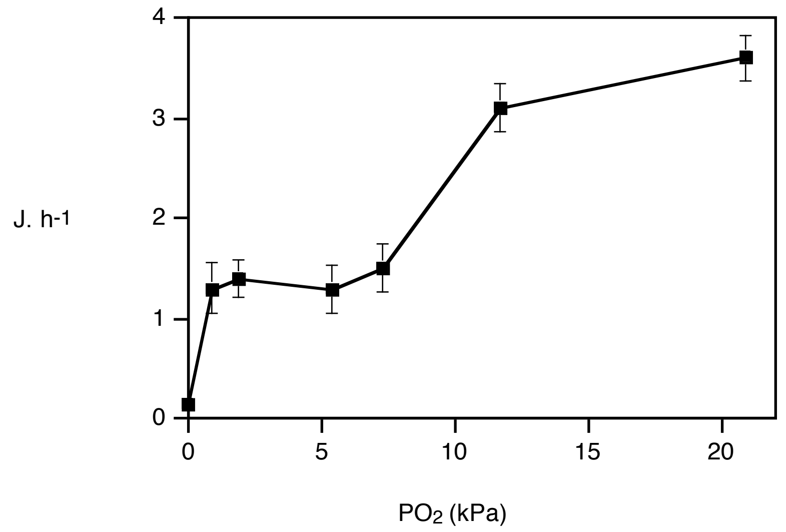

Fig. 3.2 - Metabolic rate (J. $h^{-1} \pm$ SE) of *Ruditapes decussatus* in response to declining oxygen tensions (kPa).

The oxygen utilization (extraction) efficiency and oxygen availability was calculated using the equation:

$$E = VO_2 / (VR \times \mu M\ O_2),$$

where E is the extraction efficiency or the amoumt of O_2 removed from the water, VO_2 is the oxygen uptake (µmoles O_2. h^{-1}), VR the ventilation rate (= clearance rate) in l. h^{-1}, µM O_2 is the oxygen concentration in the water. VR x µM O_2 is the total oxygen made available to the clams by the ventilatory currents (µmoles O_2. h^{-1}).

Extraction efficiency is very low (*c.* 0.3 %) and relatively constant down to 3 kPa and then increases to > 10 % under extreme hypoxia (Figure 3.3). Oxygen availability decreases steadily with declining PO_2 because there is no marked compensatory increase in ventilation rate.

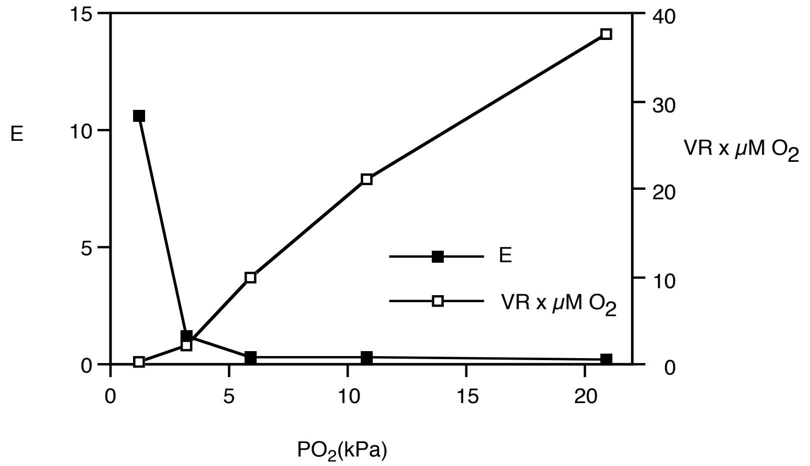

Fig. 3.3 - Oxygen extraction efficiency (E), amount of oxygen made available (ventilation rate x μM O_2) to *Ruditapes decussatus* under normoxia and different hypoxia levels.

Convection requirement, or the volume of water pumped per unit O_2 consumed (VR / VO_2), increases to a maximum at *c.* 6 kPa, higher ventilation rates being needed to extract the limited amount of oxygen in the water at lower PO_2 values (Figure 3.4). However, the clam is unable to increase or maintain ventilation rate below *c.* 6 kPa and it has to lower metabolic rates as the convection requirement declines under these extreme hypoxic conditions.

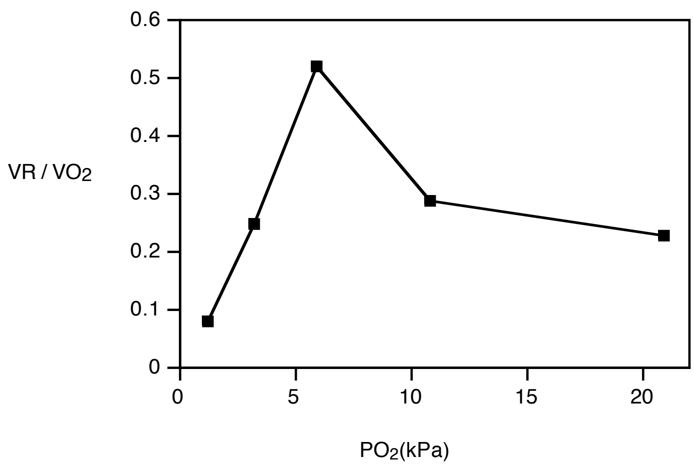

Fig. 3.4 - Convection requirement (VR / VO_2) for *Ruditapes decussatus* under normoxia and at different levels of hypoxia.

3.3.4. Scope for growth (SFG)

Scope for growth (J. h^{-1}), is calculated for conditions ranging from hypoxia to anoxia, the components being summarized in Table 1. Scope for growth under normoxia was performed at a slightly higher algal cell concentration than the other hypoxia experiments, this resulting in a higher value. Under moderate levels of hypoxia (i.e. 11 kPa) there is evidence of a relatively constant ingestion and metabolic rate (Figs. 3.1 and 3.2), consequently scope for growth (Table 3.1), under normoxia is considered to be the same as under moderate hypoxia. Growth rate is generally maintained down to *c.* 6 kPa, but becomes markedly reduced below this PO_2 (Figure 3.5). At 1.2 kPa scope for growth is 12 % of normoxic rates.

Table 3.1. Components of the energy budget (J. h^{-1} ± SE) of *Ruditapes decussatus* (0.3 g dw) under hypoxia (kPa). C - energy consumed, AE - absorption efficiency, A - energy gain from food, R - energy loss through respiration, SFG - scope for growth.

PO_2 for C (kPa)	PO_2 for R (kPa)	**C** J. h^{-1}	**AE** %	**A** J. h^{-1}	**R** J. h^{-1}	**SFG** J. h^{-1}
11	12	39.1 ± 4.2	34.6	13.5 ± 1.4	3.1 ± 0.3	21.3
6	7	47.8 ± 3.8	36.9	17.7 ± 1.4	1.5 ± 0.3	21.3
3	5	14.8 ± 3.8	51.6	7.6 ± 2.0	1.3 ± 0.2	3.7
1.2	0.9	6.4 ± 1.2	69.3	4.4 ± 0.9	1.3 ± 0.3	2.6

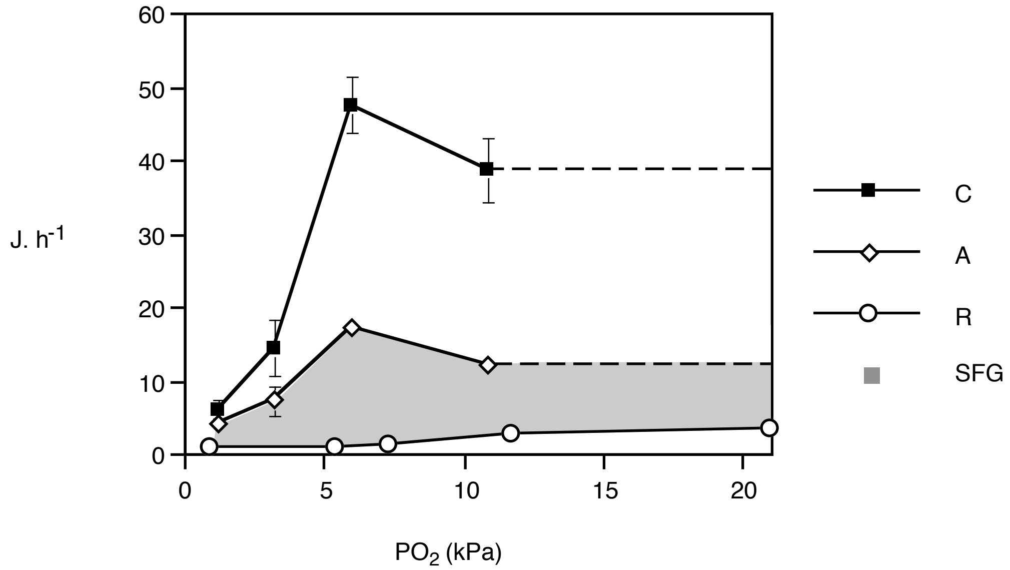

Fig. 3.5 - Partitioning of the energy budget (J. h^{-1}) of *Ruditapes decussatus* under normoxia and hypoxia. C - energy consumed, A - energy absorbed, R - energy respired, shaded area - SFG.

3.4. Discussion

Ruditapes decussatus, like most bivalves (reviewed by Bayne & Newell, 1983) is able to maintain respiration rates independent of declining oxygen down to *c.* 11 kPa, or 50 % of air saturation.

R. decussatus responds to further increases in hypoxia by lowering its respiration rate. The slight increase in clearance (= ventilation) rate at 11 kPa suggests some physiological compensation mechanism allowing the clams to increase pumping of water in order to make more oxygen available under moderate hypoxia, though this is more marked in other infaunal species (Taylor & Brand, 1975, Brand & Morris, 1984, Wang & Widdows, 1993a).

Both clearance rates and ingestion rates (Figure 3.1) decline below 6 kPa, indicating not only a decrease in ventilation but also feeding activity. However, complete cessation of clearance rate did not occur even at oxygen tensions as low as 1.2 kPa. At these very low oxygen tensions clams showed narrow opening of the valves and siphons, although they continued to ventilate.

At the intermediate PO_2 levels between 12 and 6 kPa the clearance and respiration rates do not show a parallel or coupled response to declining oxygen tensions. The rate of oxygen uptake declines below 11 kPa indicating the inability of the clam to regulate and maintain its respiration rate.

As oxygen availability declines with increasing hypoxia then extraction efficiency increases. Extraction efficiency is very low and more or less constant (Figure 3.3) throughout most of the range tested, only at the lowest level of hypoxia does it increase significantly, thus indicating some ability to increase the efficiency at the lowest oxygen levels. This very low oxygen extraction efficiency is related to the high clearance rates of suspension feeding bivalves, which typically pump more water than they require for respiratory purposes. According to Jørgensen *et al.* (1986) bivalves that live in coastal environments can process more than 15 l of water for each ml of oxygen consumption without this implying an important energetic cost. Due to the low cost associated with the ciliary pump of bivalves gills (Jørgensen *et al.* 1986; Widdows & Hawkins 1989) clearance rate (or ventilation rate) can be maintained down to lower PO_2 levels (below 6 kPa) compared to respiration rate which has a critical PO_2 of *c.* 12 kPa.

Water convection requirement thus increases as PO_2 declines to 6 kPa. However, in spite of the low costs of the gill pump, bivalves appear unable to maintain high ventilation activity at lower PO_2 values and the convection requirement declines under extreme hypoxia (Figure 3.4).

Oxygen critical partial pressure (Pc) is *c.* 12 kPa, representing 56 % of full air saturation (normoxia), and comparable to the Pc value reported by Wang & Widdows (1993b) for *Mytilus edulis.* According to Herreid (1980), Pc varies with environmental conditions and the physiological state of the animal, depending on metabolic demands and upon the ability to supply oxygen to the tissues (ventilation, diffusion area) and so should be mostly an indicative of when the metabolic rate is significantly depressed compared to normoxia.

Respiration rates of R. decussatus exposed to PO_2 values between 1 and 7 kPa are maintained at a relatively low and constant rate (36 % of normoxia) (Figure 3.2), indicating some regulation at these levels, thus enabling the clam to survive periods of hypoxia which are likely to occur in its typical habitat.

Anaerobic metabolism, as measured by heat dissipation under anoxia, accounts for only 3.6 % of the normoxic rates measured as oxygen consumption. The low anoxic rate, and thus reduced energy expenditure represents an energy saving mechanism (Widdows & Shick, 1985). Sessile and infaunal bivalves generally show a strong resistance to anoxia due in part to a reduction in metabolic activity and energy utilization (Widdows, 1987). Low values of the ratio anoxic : normoxic rates have been found for epifaunal species including *M. edulis* (Wang & Widdows, 1993b), *Modiolus demissus* (Pamatmat, 1983), *Crassostrea virginica* (Widdows *et al.* 1989) and infaunal species like *Abra tenuis* (Wang & Widdows, 1993a)

As simultaneous calorespirometry was not performed in this study the contribution of anoxic metabolism to the total metabolism under normoxia and different hypoxia levels is unknown. However, according to the low metabolic rate observed under anoxia the anaerobic component of total metabolism is likely to be absent or be very small throughout most of the range of hypoxia, as it is for other bivalve species whose strategy is also to lower their metabolism under oxygen stress. For thermodynamical and biochemical reasons any major involvement in anaerobiosis to compensate for the decline in aerobic metabolism is costly in terms of substrate utilization (Widdows, 1987, Gnaiger *et al.* 1989). An increased glycolytic flux in order to maintain anaerobic ATP turnover rates, known as the "Pasteur effect", would be very costly and is absent in bivalves (De Zwaan & Wijsmann, 1976; Shick *et al.* 1986).

Nevertheless very low anaerobic metabolic rates allow survival under periods of anoxia, which are regularly experienced by intertidal bivalves. Wang *et al.* (1992), found that *M. edulis* had an MMT (median mortality time) of 9.6 days under anoxia (N_2) at 15 °C, De Zwaan *et al.* (1991), recorded an MMT for *Mytilus galloprovincialis* and *Venus gallina* under anoxia (N_2) of 16 days at 20 °C and 4 days at 18 °C, respectively. Widdows & Shick (1985) air exposed *Cardium edule* for 5 hours and found that the cockles can switch to air-breathing (by gaping) after an initial period of anaerobic metabolism. *R. decussatus* also gapes and survives aerial exposure for a period of up to 2 days at 20 °C and anoxia (N_2) for at least 24 hours without mortality. The MMT at 20 °C for air exposed *R. decussatus* is *c.* 3 days (Sobral, unpublished).

Scope for growth is greatly diminished, *c.* 14 % normoxia, under extreme hypoxia (Figure 3.5), but does not appear to be negative, indicating that even under low oxygen tensions, clams are not having to utilize their energy reserves. It appears that the aerobic metabolic rate between 0.9 and 7 kPa probably represents the maintenance cost for the clams, *c.* 34 % of the total metabolic costs under normoxia. In *M. edulis* maintenance costs have been shown to be about 50 % of the total metabolic costs under normoxia and at ration level *c.* 6 times the maintenance ration (Widdows & Hawkins, 1989). In *M. edulis* larvae (>155 µm) supression of costly processes of feeding and growth occurred under hypoxia below *c.* 6 kPa (i.e. <50 % of normoxic rates) but no oxygen-independent metabolism was found below 6 kPa (Wang & Widdows, 1991). The ability to reduce metabolic costs, but still meet the maintenance costs by aerobic catabolism, enables *R. decussatus* to tolerate hypoxic conditions which occur particularly in the summer in the sediments.

The scope for growth calculated in this study is unlikely to provide a measure of the growth potential under more extreme hypoxic conditions because the physiological responses were not measured under long-term steady state conditions. Rather, they reflect the clams ability to tolerate and maintain their growth potential under short term and transient conditions of hypoxia which may occur at periods during the summer.

R. decussatus therefore seems to be well adapted to hypoxic environments, maintaining some feeding and digestive activity, albeit at low rates, and lowering its metabolic rate and thus conserving energy in order to survive periods of hypoxic and anoxic stress.

4. EFFECTS OF ELEVATED TEMPERATURES ON THE PHYSIOLOGICAL RESPONSES OF *Ruditapes decussatus* AND RESISTANCE TO AIR EXPOSURE.

4. Effects of elevated temperatures on the physiological responses of *Ruditapes decussatus* and resistance to air exposure.

4.1. Introduction

Temperature is a very important factor limiting bivalve distribution, both latitudinal / geographical and littoral, affecting their activity level and energy balance. Geographical distribution thus reflects the thermal tolerance of an animal.

Within a species there are obvious differences in the temperature range that can be tolerated by animals inhabiting different geographical areas. Studies at the biochemical level have shown that the reason for this variation is the genetically determined enzymatic proteins primary structure (Hoffmann, 1983). The compromise between thermostability and regulation of the specific catalytic efficiency of enzymes is the basis of the evolutionary adaptation to temperature.

According to Hoffmann (1976), proteins from organisms that inhabit warm environments have a greater resistance to heat than those of organisms from cold habitats. Therefore, thermal tolerance declines with increasing latitude.

4.1.1. Temperature tolerance and compensation mechanisms

Besides genetic adaptation, temperature tolerance can also be modified by physiological and behavioural acclimation to the temperature regime of the animals environment. Ansell *et al.* (1980a) found that the upper temperature tolerance of *Tellina fabula* and *Tellina tenuis* is higher in individuals from Mediterranean populations than in individuals from from the North Atlantic. Ansell *et al.* (1980b,1981) also found that *Donax trunculus* and *Cerastoderma glaucum*, both from shallow waters have a greater thermal tolerance to high temperatures than *Donax vittatus* and *Cerastoderma tuberculatum* from deeper waters. Acclimatory processes are responsible for field adaptation (e.g. position on the shore), acclimation to seasonal temperature fluctuations, or thermal acclimation in the laboratory.

The effects of temperature change on the metabolic processes and activity can be described as “acute” when resulting from a short-term change in temperature, or “acclimatory” responses when involving longer term compensatory adjustments of the metabolic functions.

Mechanisms involved in acute temperature compensation are enzyme-substrate interactions, such as the nearly instantaneous increase in the ability to bind substrate as the temperature is lowered, reported by Hochachka & Somero, (1973), *in* Hoffmann (1983). Although it has not been investigated, there is some evidence consistent with the hypothesis of positive thermal enzyme modulation being important in effective immediate metabolic temperature compensation.

According to the same authors, this process is also involved in acclimation processes, when temperatures are above the temperature of minimal enzymatic reaction rate there is a direct relationship between ambient temperature and the rate of reaction (positive thermal modulation) which is responsible for temperature independence of catalytic function.

Acclimatory (long-term) responses are characterized by marked changes in the level of protein synthesis, quantitatively leading to changed concentration of the same enzyme and so altered specific function , and qualitatively leading to induction of thermo-isoenzymes (Churchill, 1987 and Ramos-Martinez & Torres, 1985, respectively, *in* Hawkins & Bayne, 1992).

The temperature-induced synthesis of specific isoenzymes within well defined thermal limits has been reported in mussels, *Mytilus edulis* (Livingstone & Bayne, 1974 *in* Hoffmann,1983) and *Choromytilus meridionalis* (Seiderer & Newell, 1979 *in* Hoffmann, 1983), and according to Hoffmann (1983), alterations in the concentrations of temperature sensitive inorganic divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}), may also cause a certain degree of modulation of enzyme activity. Moreover, it is well known that such cations are also required as cofactors for many regulatory enzymes in molluscs.

Several factors can modify the acute temperature response, namely the exposure temperature, the thermal history of the animal, its body size, reproductive condition and metabolic level. Tolerance to temperature varies also with time of exposure and a certain degree of adaptation is usually demonstrated following continued exposure to a near lethal temperature, thus generally extending the tolerance limit (Schmidt-Nielsen, 1990).

4.1.2. Temperature regulation

Animals living in the intertidal zone are exposed not only to seasonal fluctuations but also to wide short-term (12 - 24 hours) variations of temperature (i.e. 10 - 25 °C) and are able to maintain their feeding and respiratory rates relatively independent of these fluctuations in environmental temperature (Bayne *et al.*, 1976). Feeding inhibition and hypoxia (Newell & Bayne, 1973), with which intertidal bivalves have to cope during the emersion period are also reflected in long-term changes of metabolic response to temperature.

Widdows (1976), working with *M. edulis* has shown that for cyclic temperatures of 6-14 °C and 11-19 °C the process of thermal compensation takes *c.* 14 days and clearance and respiration rates become increasingly independent of the temperature within the range of the fluctuating regime. This author has also shown that adaptation to 12 hours cyclic temperatures (between 21-29 °C) causes respiration in *M. edulis* to be temperature independent over that range and extends the zone of activity and thermal tolerance.

Wilson & Elkaim (1991) reported regulation of respiration by *Macoma balthica* from mid-shore and high-shore groups from northern France within the temperature range of 15-30 °C.

Despite the evidence of adaptation processes, elevated temperatures will ultimately lead to death when compensation adjustments are no longer possible and disruption of vital functions occur. Heat death is caused mainly by different temperature effects on interdependent enzymatic reactions and effects on membrane structure (Schmidt-Nielsen, 1990), but the mechanisms leading to the modification of the lethal limit are not yet clearly understood.

Maintaining metabolic activity independent of temperature changes is of great ecological significance in situations of environmental stress, involving extreme fluctuations in temperature, during starvation, or when exposed to air at low tide, as it helps to conserve metabolic energy reserves.

In terms of energy conservation and considering temperature variations, organisms would be expected to make compensatory adjustments to both components of energy gain (feeding processes) and energy loss (through respiration and excretion) in a way that the balance between both components remains positive, still enabling growth and reproduction (scope for growth). Acclimatory adjustments in metabolic energy expenditure thus appear to be linked primarily with conservation of energy resources (Bayne & Newell, 1983).

4.1.3. Resistance to air exposure

Exposure to air at low tide enhances high temperatures at the sediment level which together with desiccation, the inability to feed and the lack of an oxygen supply are certainly stress factors reducing scope for growth of infaunal bivalves, even if behavioural adaptations, like burying deeper in the sediment, may help to reduce exposure to extreme temperatures.

The ability of utilizing oxygen from air is widespread among intertidal molluscs, but the patterns of activity under air exposure vary between species (see Bayne & Newell, 1983), reflecting the varied degrees of adaptation to aerial conditions, such as type of gill and degree of vascularization of the mantle cavity.

Widdows *et al.* (1979), have shown that the ability of intertidal bivalves to maintain aerobic metabolism in air depends on behavioural responses such as intermittent air-gaping. The epifaunal *M. edulis* and *Mytilus* *galloprovincialis* do not gape, accumulating anaerobic metabolism end products when exposed to air, thus avoiding desiccation and saving energy. The infaunal bivalves *Cerastoderma edule* and *Modiolus demissus* are not faced with desiccation and are able to remain partially open and extract oxygen from air. Widdows & Shick (1985) found that *C. edule* maintains a fully aerobic metabolism when exposed to air, without resort to major utilization of reserves through anaerobiosis. *Cerastoderma glaucum*, a subtidal species, does not gape and is less able to tolerate air exposure than *C. edule*, reflecting its inability to colonize higher-shore levels (Boyden, 1972).

Though many studies have examined the influence of temperature acclimation on specific rate functions in bivalves (reviewed by Bayne & Newell, 1983), little attention has been given to the integration of the different functions at the level of the whole organism. In particular there is no information concerning the infaunal bivalve *R. decussatus* and its integrated physiological responses to temperature variation.

R. decussatus living in the tidal flats of the Ria Formosa, experience elevated temperatures (up to *c.* 30 °C in the exposed sediments), during summer. The effects of these elevated temperatures on the clams physiological performance are unknown, and the possible contribution of high temperatures to the high clam mortality reported in recent years remains uncertain.

Therefore, this study aims to assess the acute response of *R. decussatus* to temperature change through the use of physiological energetics (i. e. scope for growth) and also to examine the effect of elevated temperature on the resistance to air exposure.

4.2. Materials and methods

Clams were collected from the sampling site in Ria Formosa, at low tide in August 1994, and allowed to acclimate (for c. 4 days) to laboratory conditions (15 °C and 35.6 ‰) at the Departamento de Ciências e Engenharia do Ambiente in Lisbon. Mean length of clams was 32.4 ± 0.2 mm (n = 23). They were fed with a culture of *Phaeodactylum tricornutum*, twice daily and the water was changed every two days.

Experiments were performed at 20 ± 1, 27 ± 1 and 32 ± 1 °C in a controlled temperature room. Clams were allowed to acclimate to the experimental temperature for 2 days prior to physiological measurements.

Clearance rates, respiration rates and absorption efficiency measurements and the calculation of scope for growth were performed in the way described in the Chapter 2. Six to seven animals were used in the experiments. As excretion may be significant at high temperatures (Bayne *et al.*, 1976, Bayne & Scullard, 1977), ammonia excretion rate was measured on 7 to 9 animals by the phenol-hypochlorite procedure described by Solórzano (1969).

Energy equivalents used for physiological measurements were 1 µmol O_2 = 0.456 J (Gnaiger, 1983); 1 µmol $N\text{-}NH_4$ = 0.349 J (Elliot & Davidson, 1975) and 1 mg POM = 23 J (Widdows *et al.*, 1979). Significance of differences in rates at the three experimental temperatures were statistically analysed by means of a two-sample t-test.

Resistance to air exposure was performed by maintaining two groups of

20 clams air exposed in controlled temperature rooms, one at 20 ± 1, and other at 28 ± 1 °C. Another set of 20 clams were air exposed in an oven at 35 °C. Clams were checked daily and considered dead when there was no closing of the valves after stimulation.

4.3. Results

The physiological responses (clearance rate, respiration rate and ammonia excretion rate) of *R. decussatus* exposed to 20, 27 and 32 °C are shown in Figure 4.1.

Clearance rate declined with increasing temperature and at 32 °C was *c.* 60 % of the rate observed at 20 °C. All clearance rates were significantly different ($P < 0.01$, t-test) for the three experimental temperatures. An increase of *c.* 30 % was observed for absorption efficiency in the same range. Respiration rate increased slightly with temperature and differences were not significant. Excretion rate showed little change with increasing temperature and differences were also not significant.

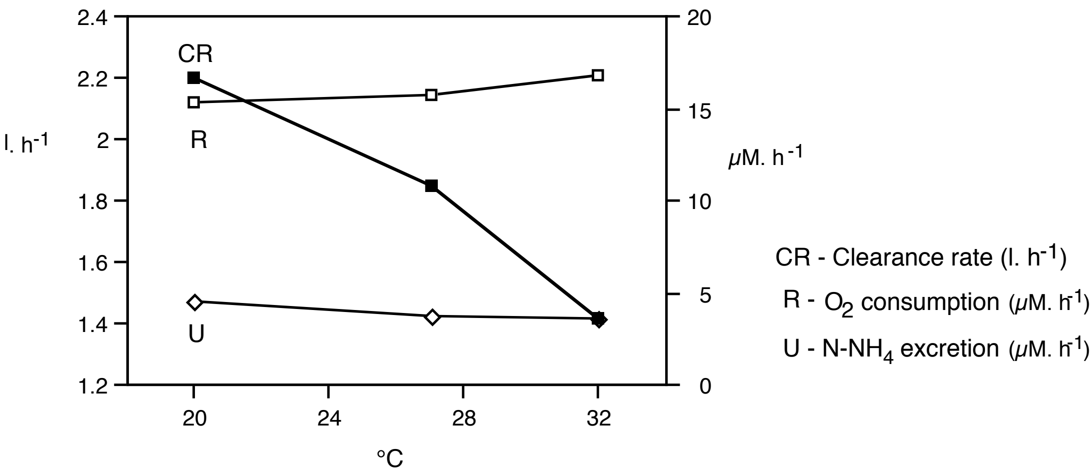

Fig. 4.1 - Physiological responses of *Ruditapes decussatus* acclimated to 15 °C and exposed for 2 days at 20, 27 and 32 °C.

Temperature quotients (Q_{10}) were determined for respiration rates and were found to be 1.04 for the 20 to 27 °C interval and 1.13 for the 27 to 32 °C interval (1.07 from 20 to 32 °C).

Scope for growth declined with increasing temperature between 20 and 32 °C. At 27 °C it approached zero and then became negative at the highest temperature of 32 °C (Figure 4.2). The components of the energy budget (J. h^{-1}) are summarized in Table 4.1.

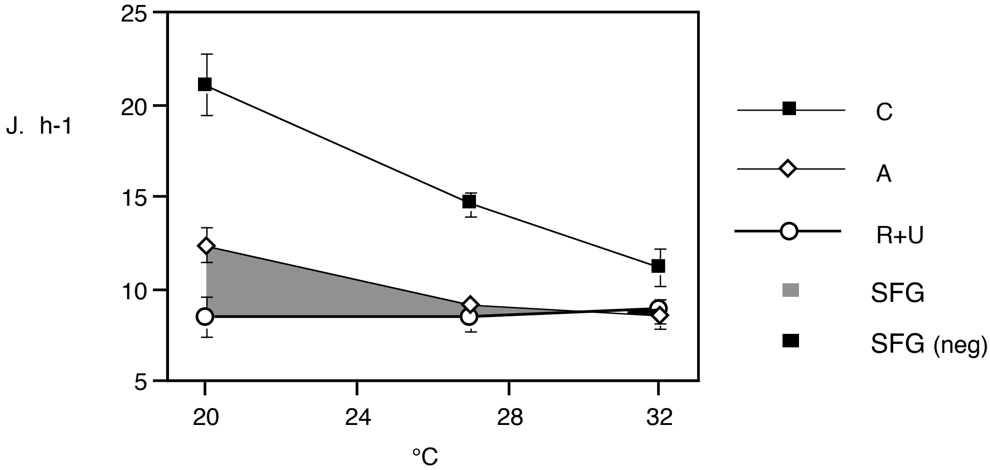

Fig. 4.2 - Components of the energy budget (J. h^{-1}) of *Ruditapes decussatus* acclimated to 15 °C and exposed for 2 days at 20, 27 and 32 °C. C - energy consumed, A - energy absorbed from food, R - energy loss through respiration, shaded area - scope for growth (SFG), black area - negative SFG.

When clams were air exposed to three temperatures (20, 28 and 35 °C), mortality was faster at the highest air temperature (35 °C). All clams started gaping after 1 to 2 hours and died in less than 24 h (Figure 4.3). At 20 and 28 °C they started gaping after 4 hours and it took 4 and 5 days respectively, to reach 100 % mortality. At 20 °C no mortality occured

Table 4.1. Components of energy budget (J. h^{-1}) for 15 °C acclimated *Ruditapes decussatus* standard weight of 0.3 g dw after 2 days exposure to 20, 27 and 32 °C. C - energy consumed, AE - absorption efficiency, A - energy absorbed from food, R - energy loss through respiration, U - energy loss through excretion, SFG - scope for growth.

Temp °C	**C** J. h^{-1}	**AE** %	**A** J. h^{-1}	**R** J. h^{-1}	**U** J. h^{-1}	**SFG** J. h^{-1}
20	21.2	58.8	12.5	6.9	1.6	3.9
27	14.7	64.6	9.2	7.2	1.3	0.7
32	11.3	77.1	8.7	7.6	1.3	-0.3

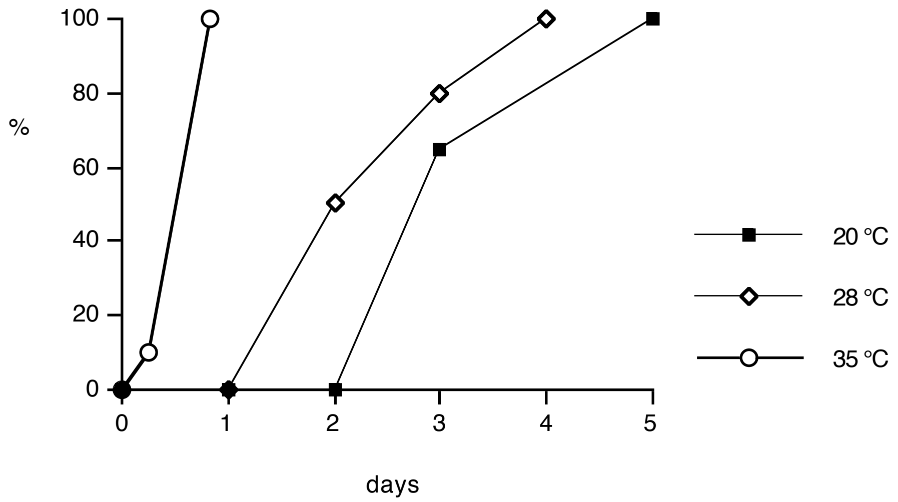

Fig. 4.3 - Percentage mortality of *Ruditapes decussatus* air exposed to different temperatures.

for 2 days. Lethal times in air for 100 % of the clams (LT_{100}) are therefore 120 hours at 20 °C, 96 hours at 28 °C and 20 hours at 35 °C. LT_{50} taken from the mortality curves are *c.* 3 days at 20 °C, *c.* 2 days at 28 °C and *c.* 10 hours at 35 °C.

4.4. Discussion

The increase in temperature over the range 20 - 32 °C caused a marked reduction in the scope for growth of *R. decussatus*. Scope for growth declined approximately to zero values above 27 °C and was negative at 32 °C. This was mainly due to the lowering of the energy input (i.e. food consumption) as a result of a decline in feeding rate and this probably reflects the disruption of temperature adaptation mechanisms and inhibition of feeding activity at elevated temperatures as found for species of *Crassostrea*, *Ostrea*, *Mytilus* (Ali, 1970, Walne, 1972, Winter, 1976, Widdows, 1978a, Newell & Branch, 1980). High temperatures (above 27 °C) are thus stressful to the clams as shown by the low and even negative values of SFG.

Generally respiration rates increase with temperature up to a sub-lethal level where activity and metabolic processes are disturbed preceeding lethal effects. It is also known that compensatory adjustments can be made by the animals in order to cope with changes in temperature. Such acclimatory processes depend on the time-course, the amplitude and the rate of temperature change, the average value about which fluctuations occur, as well as the thermal history of the animals, their size and reproductive condition and their geographical distribution (Bayne *et al*., 1977). Related to the rate of temperature change are factors like season, position on the shore and depth to which the animals burrow (Wilson & Elkaim, 1990).

To emphasize the importance of previous thermal history and/or latitudinal/genetic differences on bivalve temperature tolerance Figure 4.4 was redrawn from Bayne *et al*. (1977) and modified to allow data from the present study to be plotted.

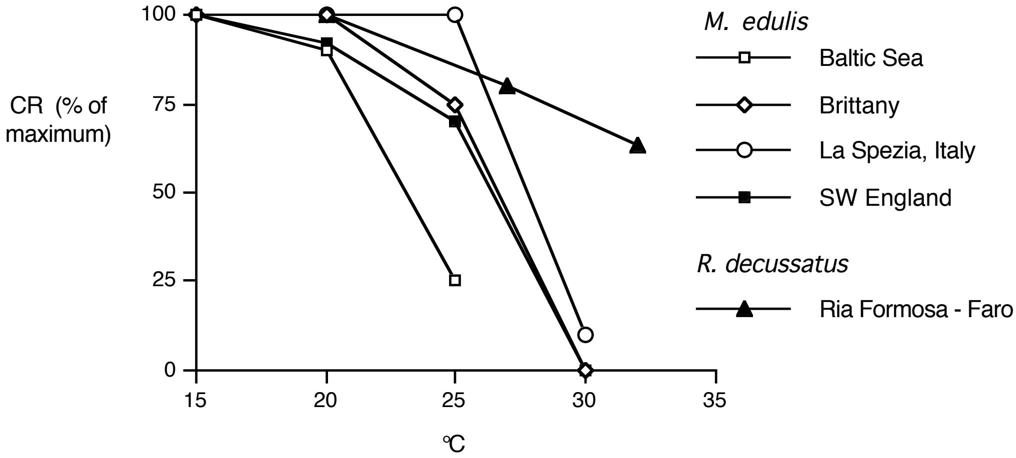

Fig. 4.4 - Clearance rates of *M. edulis* from the Baltic Sea, Brittany, La Spezia, Italy and SW England and *R. decussatus* from Ria Formosa, Faro, at different temperatures (adapted from Bayne *et al.*, 1977).

Clearly bivalves adapted to higher fluctuating summer temperatures, such as clams from the Ria Formosa lagoon, and mussels from more southerly latitudes, have extended their tolerance zones and can keep their filtration rates to *c.* 50 % at high temperatures (32°C). The geographical component of the thermal tolerance is also evident with northern populations exhibiting lower tolerance to warmer temperatures than southern populations which experience these higher environmental temperatures as shown by Ansell *et al.* (1980a) for *Tellina fabula* and *Tellina tenuis* is from North Atlantic and Mediterranean populations.

This pattern is reflected in a typical translation of the rate : temperature curves to the right (Fig 4.4) indicating compensation adjustments and / or anti-clockwise rotation (Hoffmann, 1990), due to lower temperature coefficients (Q_{10}) and thus better regulation of metabolism at warmer temperatures.

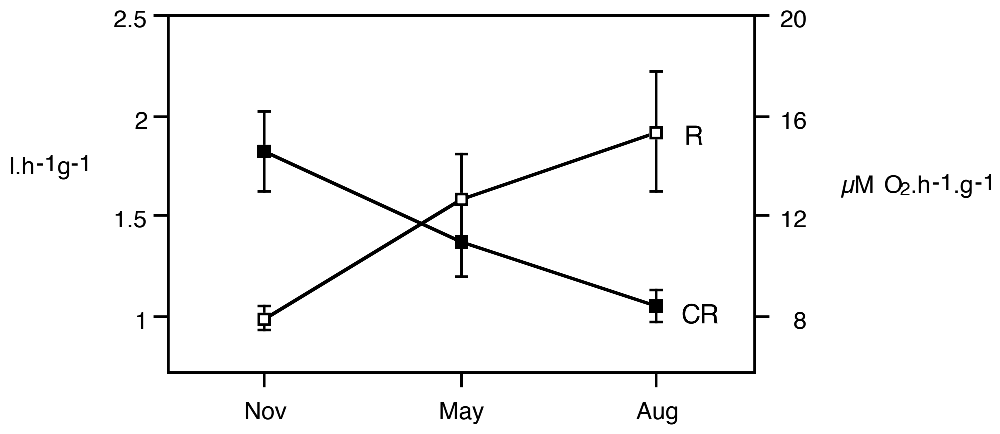

Fig. 4.5 - Seasonal variation of clearance rate CR (l. $h^{-1}g^{-1} \pm$ SE) and respiration rate R (µM O_2. $h^{-1}g^{-1} \pm$ SE) of *Ruditapes decussatus* acclimated for 48 hours at 20 °C.

Evidence of the seasonal relationship between temperature and the clearance and respiration rates of *R. decussatus* is shown in Figure 4.5 where previous data are combined. As complete acclimation is not likely to have occured after 48 hours exposure, these data probably reflect an intermediate level between the acute response and the fully acclimated response which may take 14-16 days in bivalves (Widdows, 1976). Consequently, in November the experimental temperature (20 °C) will be above the field temperature (c. 12 °C), and in August the experimental temperature will be lower than the field temperature (*c.* 24 °C).In May environmental and experimental temperatures were similar and the results probably reflect true environmental rates. Experimental food levels in the experiments relative to environmental food levels may also modify the relationship between feeding rate and temperature.

The same type of temperature dependent response after 2 days exposure was found by Widdows & Bayne (1971) for clearance and respiration rates of the mussel *M. edulis* from SW England. However the respiration of *R.*

decussatus appears to follow an opposite pattern, increasing from November to August when they are subjected to a reduction in temperature (environmental temperature of *c.* 24 °C to experimental temperature of 20 °C). This seasonal increase in respiration probably reflects the increase in metabolic costs associated with production, both somatic and gametic.

Through acclimation, physiological rates can be maintained relatively temperature independent and tolerance is enhanced allowing improved survival in an extended temperature range. Widdows (1976) showed that when *M. edulis* where subjected to temperature which extended above their normal environmental maxima (21 to 29 °C), they showed a temperature-independent respiration rate over the entire range, and an extension of the zone of activity and temperature toolerance. Also for *M. edulis* Widdows & Bayne (1971) demonstrated that complete acclimation of oxygen consumption and filtration rates occurs within 14 days, with either cold or warm acclimated animals.

In the present study where clams were acclimated for two days at each of the experimental temperatures a significant decline in feeding rate was observed with increasing temperature, but this was partially compensated by an increase in the absorption efficiency, so that negative scope for growth only occurs at the highest temperature (32 °C). The decrease in scope for growth nevertheless indicates that raising temperatures constitute a physiological stress for the clams.

A similar effect was recorded in *M. edulis* where the SFG declined following a temperature increase and during the process of warm acclimation (Widdows & Bayne, 1971). Therefore both clams and mussels show physiological stress during warm acclimation due to the different effect of temperature on clearance rates (suppression) and respiration rate (enhancement).

According to Walne (1972), experiments in the UK demonstrated that *R. decussatus* is very sensitive to reductions in temperature from 20 to 10 °C, and lowers its filtration rate by more than 45 %. Therefore it appears that the optimum temperature for *R. decussatus* is around 20 °C as its physiological performance is poorer at lower and higher temperatures, due mainly to reduced filtration rates (*c.* 45 % at 10 °C and *c.* 25 % and *c.* 40 % at 27 °C and 32 °C, respectively).

A slight increase in the respiration rates was observed but according to the value of Q_{10} (1.07) we can say that respiration rate is relatively independent of temperature within the range studied (20 - 32 °C). Intertidal bivalves experience a wide range of diurnal and seasonal temperature fluctuations and adaptation to these fluctuations causes respiration to be temperature independent extending the thermal tolerance.

Bodoy *et al.* (1986) in France, found a maximum respiration rate at 25 °C for unfed 7 mm *R. decussatus,* but this relationship can vary with the physiological conditions and seasonal thermal acclimation. The same author found that this species is not as sensitive to high temperatures as *Ruditapes philippinarum,* a related species. This insensivity is certainly related to seasonal acclimation processes and it is expected that southern European populations of *R. decussatus* like those in Ria Formosa are adapted to high environmental temperatures specially during summer, when this experiment was performed. The ability of *R. decussatus* to maintain its metabolic rate relatively temperature independent between 20 °C to 32 °C as demonstrated in this study illustrates this point.

As we can see the physiological energetics approach provides an integrated and sensitive measure of the condition of clams exposed to high temperatures. Respiration rates or clearance rates alone cannot account for temperature stress in the range studied (20 - 32 °C).

Mass mortality of *R. decussatus* has been reported during the summer months in the Ria Formosa since 1983. The results of this study indicate that elevated temperature is unlikely to be the primary factor causing the mass mortality. It is probably caused by a combination of environmental stress factors, namely, high temperature, poor body condition, low reserves following spawning and parasitic infestation of clams.

In August the spawning period is at its end, and the clams reserves are only starting to build up. Consequently it is a time of year were the clams may be more “fragile” and sensitive to extreme environmental conditions. Widdows (1978b) also found lower tolerance to temperature stress for *M. edulis* in the post-spawning period.

Ruano & Cachola (1986) and Ruano (1989) have reported that clams from Ria Formosa are infected by several parasites the most widely distributed and prevalant being *Perkinsus atlanticus*. This agent can cause a 60 % reduction in the gill area, potentially affecting its feeding and respiratory function. According to Vigário & Ruano (1990) temperature seems to be the most important factor controlling the development of this parasite and summer temperatures and salinity in the Ria Formosa are ideal for its development.

While it is beyond the scope of the present study, it is likely that the presence of parasites, namely *P. atlanticus*, can affect the physiological performance of *R. decussatus,* but to what extent remains unknown.

Environmental stress, natural or anthropogenic, may compound the damage caused by parasitism and further compromise the ecological fitness of the clams.

Differences in temperature tolerance limits in air were not marked between 20 and 28 °C (LT_{50} *c.* 2 and 3 days, respectively), but at 35 °C half the population died within 10 h. Gaping indicating stress, was observed at all temperatures leading to a faster aerobic metabolism and the rapid utilisation of energy reserves, desiccation and consequently death.

The chance of meeting extremely high temperatures in the field for long periods is low, it may occur at some higher intertidal levels which are exposed during neap tides, but the cumulative effect of exposure to high air temperatures over many days/tidal cycles is unknown.

Clams can also show a behavioural response and bury deeper into the sediment to avoid the near surface higher temperatures (up to 29 °C, Sobral, unpublished). According to Lent (1969), *in* Bayne *et al.* (1976) evaporative cooling of the mud reduces the microhabitat temperature by 5 to 10 °C and helps to reduce desiccation as the relative humidity in the mud/air interface is nearly 100 %. Burying deeper poses other problems as the sediment can be hypoxic and anoxic and reduced conditions can give raise to sulphide release from the sediments (Roden & Tuttle, 1992). In fact, Madureira *et al.* (1994) have found specially high levels of H_2S and FeS in the superficial sediments in the Ria Formosa particularly in the summer. According to these authors the high levels of H_2S, which are toxic to many aquatic animals, can probably affect the growth of clams in the Ria.

High temperatures (i.e. above 28 °C) can therefore significantly weaken clams resistance to air exposure and other stresssors and affect their performance in water leading ultimately to death.

5. EFFECTS OF CURRENT VELOCITY, TURBIDITY AND PARTICLE SIZE SELECTION ON THE PHYSIOLOGICAL RESPONSES OF *Ruditapes decussatus*.

5. Effects of current velocity, turbidity and particle size selection on the physiological responses of *Ruditapes decussatus*

5.1. Introduction

In coastal areas water currents are induced by river flows, tidal cycles, wind and storms and are modified by the topography. Where the freshwater input is unimportant, like in the Ria Formosa coastal system (Falcão e Vale, 1990), tidal rise and fall and its corresponding lateral water movements, is the principal process generating currents. The speed and direction of these tidal currents are influenced by the geometry of the basin and its constraining land masses (Bearman, 1989). Tidal processes associated with strong winds generating storms have a major role in the evolution of the barrier islands of the Ria Formosa system (Pilkey *et al.*, 1989).

In the natural environment water currents are of vital importance for sessile suspension-feeding bivalves because algal cells and resuspended particles are carried by the currents. Tidal currents in fact control sediment dynamics, as well as settlement, growth and feeding of benthic animals (Wildish & Peer, 1983).

It has been recognized, specially in aquaculture related studies (Walne, 1972), that current velocity is an important factor influencing bivalve growth and condition. Hydrodynamics can also be important in determining the settlement of bivalve larvae (André *et al.* 1993) and thus species recruitment.

Relatively few studies have examined the effects of current speed on marine organisms and the response pattern over a wide range of current speeds. Wildish *et al.* (1987, 1992), Wildish & Kristmanson (1985) and Wildish & Miyares (1990) have examined the feeding responses to flow velocity on *Mytilus edulis* and *Placopecten magellanicus*. The influence of flow velocity on growth was studied on *Argopecten radians* by Cahalan *et al.* (1989), on *Mercenaria mercenaria* and *Crassostrea virginica* by Grizzle *et al.* (1992) and on *Modiolus modiolus* by Lesser *et al.* (1994).

In addition to behavioural responses the clearance rate of bivalves provides a good measure of impact and is also an index of the animal's ability to maintain its feeding activity and physiological condition when exposed to currents.

Turbidity is another important factor in coastal areas, specially in confined or sheltered environments, like estuaries and coastal lagoons where fine sediment is likely to accumulate. This fine sediment is easily resuspended by tidal currents, storms and increased river flows and so contribute to increase turbidity. Turbidity and the concentration of suspended particulate matter in estuarine and coastal systems therefore show tidal, seasonal and inter-annual variation.

The pattern of water movement and the interaction between tidal currents and wave action have important consequences for the transport and distribution of sediment on the tidal flats. Once the threshold of motion is reached particle transport is achieved by the processes of rolling, when particles roll over the bed, saltation, when particles jump into the flow and land downstream, and true suspension, when particles are carried in the water column forming a suspension carpet (Dyer, 1986).

These three ways of particle transport are in relation to increasing current velocities.

The lagoon of the Ria Formosa system is largely filled with an accumulation of fine sediment in salt marshes and flood-tidal-delta sands. The large spring tidal amplitude (4m) and minor storm events can produce overwash of the barrier islands, which is the dominant process bringing material into the lagoon (Pilkey *et al.*, 1989). According to the same authors the ebb flow from the flooded areas due to its higher velocity is responsible for erosion and channel formation.

R. decussatus of tidal flats in the Ria Formosa are consequently exposed to wide variations in both water currents and turbidity. In this dynamic system resuspension of sediment is a common feature and the ability of clams to select the appropriate food particles determines their performance.

Several studies indicate that suspended bottom material is an important food source for suspension feeding bivalves such as *M. edulis* (Kiørboe *et al.* 1980, 1981; Bayne *et al.*, 1987), *Spisula subtruncata* (Møhlenberg & Kiørboe, 1981) and *Ostrea edulis* (Grant *et al.*, 1990).

Suspension feeding bivalves regulate the ingestion of particles by decreasing the clearance rate and/or by rejection of excess particles through pseudofeces formation as has been shown for several species: *M. edulis* (Kiørboe *et al.*, 1980), *Mercenaria mercenaria* (Bricelj & Malouf, 1984), *Ruditapes philippinarum* (Daou & Goulletquer, 1988), *Mya arenaria* (Grant & Thorpe, 1991),*Cerastoderma edule* (Navarro *et al.* 1992), and *Venerupis corrugatus* (Stenton-Dozey & Brown, 1994).

The ability of bivalves to select and retain suspended organic particles from the water that is pumped through the gills determines growth. As reviewed by Jørgensen (1990), bivalves fed mixtures of phytoplankton cells and silt are able selectively to reject organically-poor particles, thus achieving an enrichment of food ingested (Iglesias *et al.*, 1992). This enrichment is particularly important when bivalves are exposed to high particulate loads from resuspension of bottom sediment, having low organic content producing a dilution effect on the seston food value (Widdows, *et al.* 1979; Vahl, 1980). Bodoy & Plante-Cuny (1984), have found that the growth rate of *R. decussatus* is correlated with the primary prodution of microphytobenthos rather than with primary production in the water column, showing the importance of resuspension of algal cells from the sediment.

Ingestion is thus accomplished following particle retention by the gill according to particle size (size selection) and preingestive sorting by the labial palps, unsuitable or excess particles being conveyed to rejection ciliary tracts on the gills as has been shown for *M. edulis* (Bayne *et al.*, 1976).

Particle retention by *M. edulis* was examined from a hydromechanical point of view by Jørgensen (1981a,b, 1990) who contests the assumption of the gill acting as a simple mechanical sieve. According to Morton (1983) it seems clear that the ciliary arrangement of the gill filaments is an effective filter, though this may be aided by mucus secretion, and that the laterofrontal cirri simply moving water past the gill as suggested by Jørgensen (1981a) is not in conflict with such structures acting as a primary sieve on the gill.

Though most bivalves retain particles ≥ 4 µm diameter with 100 % efficiency according to Møhlenberg & Riisgård (1978), *Pecten opercularis* and *Pecten septemradiata* show a decrease in retention efficiency of particles < 7 µm. Thus they appear to be structurally adapted to exploit the larger suspended particles characteristic of the coarse deposits they live on. Studies on *M. edulis* (Vahl, 1972; Møhlenberg & Riisgård, 1978) have shown the ability of this species to retain particles as small as 2 µm with great efficiency. Thus there are important and apparently adaptative differences between bivalves in their retention efficiency (Shumway *et al.*, 1985).

No information was found in the literature regarding the responses of *R. decussatus* to the changing current velocities and turbidity of its natural environment. Therefore in this chapter the physiological responses of the clam *R. decussatus* to different current velocities and different turbidity levels and the efficiency of this species in retaining particles of different size from their turbid environment are examined.

5.2. Materials and methods

Clams were collected at the sampling site in Ria Formosa at low tide in June 1994, and allowed to acclimate to laboratory conditions (temperature 20 °C, salinity 32 ‰) at the Plymouth Marine Laboratory (PML), U.K., where all the experiments were performed. Clams were maintained in a flow through system and were fed with a culture of *Isochrysis galbana* at a rate of approximately 3000 cells. ml^{-1}.

5.2.1. Current velocity

A small annular flume (Figure 5.1) was used for the current velocity experiments. It was designed and constructed at PML based on the annular flume described by Fukada & Lick (1980). The flume has a 10 cm wide circular channel of 60 cm outer diameter and 30 cm depth, which can be filled with up to 60 l of water. Water can be sampled at different depths (5, 10, 15, 20 cm) via 1 mm bore tubes connected to syringes. The rotating drive plate can create free stream velocities up to 160 cm. s^{-1}.

Fig. 5.1 - Annular flume used to study the effects of current velocity on the physiological performance of *Ruditapes decussatus*.

An incorporated electromagnetic (EM) current sensor is able to quantify the free-stream velocity and velocity-height profiles for a range of current speeds. When water moves over a sediment surface a shearing force is created, caused by layers of water moving over each other in a laminar flow. The transfer of energy results in the movement of sediment and thus the shear stress provides a measure of the force acting on the sediment and determines whether or not sediment on the bottom is likely to be moved (Bearman, 1989).

To create realistic conditions, sediment (sand) was introduced in the flume to a height of 5 cm and the clams allowed to bury overnight prior to each experiment. The sediment was collected from Whitsand Bay, Cornwall, and washed several times under running water.

We used four batches of 13 clams each, and six drive plate velocities, 2.8, 14, 39, 78, 112 and 168 cm. s^{-1} corresponding to free stream velocities (at 10 cm height) of 0.6, 3, 8, 17, 34 and 36 cm. s^{-1}. Clams (mean length 34.6 ± 0.2 mm, n = 52) were buried in the sediment within the quadrant just upstream of the point of collection of water samples. Algal cells were introduced into the flume in order to obtain a concentration of approximately 15000 cells. ml^{-1}.

In each run, 30 minutes after addition of the algal cells, two water samples separated by a two minute interval, were collected for algal cells counts at three different heights (2, 10 and 20 cm above the sediment), and this was repeated every 30 minutes for 2 hours. Cells were counted with a Coulter Counter Model D, equipped with a 140 µm aperture tube. Clearance rates were calculated from the exponential decline in algal cell concentrations at the upper height, which represents the long-term mixed algal cell concentrations in the flume. Samples from lower levels reflect small scale cell depletion at lower current velocities.

Controls, without clams were run with the addition of algal cells, at 0.6 cm. s^{-1} for the three lower speeds and at 24 cm. s^{-1} for the three higher speeds. Algal settlement in the controls was very low (equivalent to < 0.25 l. h^{-1}) and was subtracted from clearance rate values for each batch. Clearance rates values were weight standardized to a 0.3 g dw animal.

Once in each run water samples were collected from all heights (six) to obtain vertical profiles of cell concentrations in order to determine algal cell depletion in the water column in relation to current velocity. Control counts (without animals), for these profiles were again made at free stream velocities of 0.6 cm. s^{-1} and 24 cm. s^{-1}.

For each run a current velocity profile, from 16 cm height to sediment level, was obtained. Measurements were taken with a Valeport (Series 800) EM current meter with a 3.2 cm discus, at 1 cm intervals or 0.5 cm intervals when changes in current velocities were noticeable (i.e. as it approached the bottom). Values at 10 cm height were used as free stream velocities. Current measurements below 4 cm were not included in the velocity profiles due to turbulence and the moving sediment effect on the probe. Due to this effect shear stress was estimated from other current meter readings and video tracking of particles (Widdows, unpublished).

5.2.2. Turbidity

To test the influence of turbidity on clearance rate we used three seston concentrations, 10, 100 and 300 mg. l^{-1}. These were obtained by adding fine surface mud to seawater to make up a solution of the desired seston concentration. Mud was collected from the banks of the Lynher river, at Whacker Quay, Cornwall, and kept cool and dark in a refrigerated chamber.

Seston concentration was monitored with an optical back scatter probe (OBS-3 D&A Instrument Co.), previously calibrated for the same mud. Calibration was achieved by fitting a regression line to the relationship between OBS readings (volts) and the concentration of suspended material (dry weight of mud in seawater, mg. l^{-1}). The procedures used for the filtration and gravimetric analysis of suspended particles are described in Appendix II.

The turbidity experiments were performed in a static system. Sixteen 2 l beakers each with one clam, plus one control beaker without an animal, were used. The mud suspension was added to each beaker and turbidity level monitored with the OBS probe. Particles were kept in suspension by continuous stirring at the bottom with a magnetic stirrer in a small Petri dish to avoid disturbing the clams. Algal cells were also added to make up a concentration of 10000 cells. ml^{-1}.

Every 30 minutes for 1 h 30 min, one 20 ml sample was taken from each beaker with a syringe and the volume of particles measured by means of a Coulter Counter Multisizer fitted with a 100 µm orifice tube. At the two highest seston concentrations dilutions (1:10 and 1:20) were made to avoid coincidence counts and blockage of the orifice tube. Samples were always stirred prior to counting.

Clearance rates, minus control values, were calculated over two time intervals and weight standardized to a 0.3 g dw animal.

5.2.3. Particle size selection

The suspended particle size selection experiments were performed using the flume (for description see above) and 13 animals buried in sediment. Two seston concentrations, 10 and 100 mg. l^{-1}, were used. They were obtained in the same manner as for the turbidity experiments.

Samples were taken from two levels, counted (by volume) with a Coulter Counter Multisizer fitted with a 100 µm orifice tube, over a wide range of channels/particle sizes (2.4 to 9.6 µm diameter) and averaged. Dilution (1:10) was needed at the higher seston concentration to avoid coincidence counts.

Size selection was quantified by examining the relative depletion of different particle size fractions over a period of 1h 30 min. The retention efficiency was calculated using the formula 1 - (t_2 / t_1), t_1 and t_2 being two different sampling times, and then expressed as % of the larger channels/particle sizes (i.e. 9.6 µm). For this purpose only particles of diameter between 2.4 µm to 9.6 µm were considered because larger particles introduce much variability into the counts, due to their relative high volumes and very low numbers.

5.3. Results

5.3.1. Current velocity

Figure 2 shows that the maximum clearance rate (*c.* 2.5 l. h^{-1}. ind^{-1}) occurs at the lower current velocities (i.e. up to *c.* 8 cm. s^{-1}) and declines with increasing velocities, specially above 17 cm. s^{-1}. At velocities of *c.* 24 cm. s^{-1} it shows a 50 % decrease. A significant linear relation between clearance rates (l. h^{-1}) and free stream velocities (cm. s^{-1}) is explained by the equation y = -0.066 x + 2.828, with r = 0.98 (n = 6).

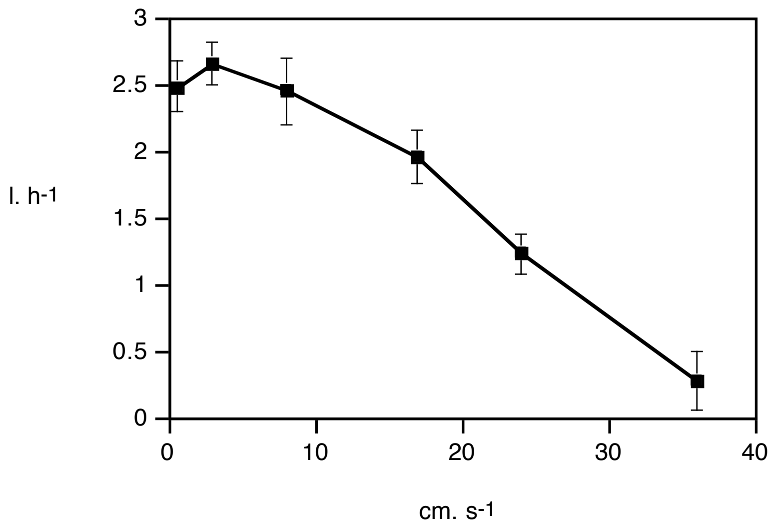

Fig. 5.2 - Variation of clearance rate (l. $h^{-1} \pm$ SE) of *Ruditapes decussatus* with free stream velocity (cm. s^{-1}) in the annular flume.

Clearance rates are depressed at shear stresses of 0.89 Pa to 1.63 Pa (Table 5.1), which correspond to free stream velocities of 17 to 36 cm. s^{-1}. These free stream velocities account for current velocities of 13 to 17 cm. s^{-1} at 4 cm above the sediment. The three lower velocities causing shear stresses of 0.001 to 0.11 Pa, are associated with no movement of surface sediment particles, and thus seem to be consistent with the maintenance of high clearance rates.

The sediment granulometry is obviously also related to the amount of shear stress needed to cause movement. Saltation of sediment grains occurred at 24 cm. s^{-1} (*c.* 1.6 Pa) and at 36 cm. s^{-1} (c. 3.8 Pa) the bulk sediment was moving and sand waves were forming.

Table 5.1 - Current velocities (cm. s^{-1}) and estimated shear stress (Pa) based on current meter observations and video-tracking of particles.

Current velocity (cm. s^{-1})	0.6	3	8	17	24	36
Shear stress (Pa)	0.001	0.02	0.11	0.89	1.68	3.8

At the three lower free stream velocities no movement of the sediment occurred, where as at 17 cm. s^{-1} (0.89 Pa) some resuspension of feces could be seen together with some rolling of the smaller particles on the bottom. At 24 cm. s^{-1} (1.63 Pa) resuspension of feces and smaller particles was observed and the rolling and saltation of particles on the bottom leading to the formation of small sand ripples that kept slowly moving. At this current velocity the siphons were being bombarded by particles and from time to time jetting would occur from the inhalant siphon, corresponding to the ejection of sand particles and cleaning of the mantle cavity. At a free stream velocity of 36 cm. s^{-1} (3.8 Pa), there was continuous resuspension of particles and the rolling and saltation was much more intense. Particles were being continuously resuspended and deposited further downstream, resulting in moving sand waves that kept covering and uncovering the clams. Some animals would struggle to stay in position at the sediment surface, but two individuals were carried away to the next quadrant of the flume. At this current velocity clams were experiencing continuous bombardment of the siphons with sand grains and some would keep them constricted at the tip while others would simply close their valves. On several occasions jets from the inhalant siphon could be observed, projecting sediment higher than 5 cm into the water column. This sediment was immediately carried away in suspension. At the end of the experiment strings of bound sediment particles could be seen on the sediment surface resulting from excess suspended material rejected as pseudofeces.

Vertical profiles of cell concentrations in relation to increasing free stream velocities are shown in Figure 5.3. In all flume runs with clams present and at all current velocities, the height at which the maximum cell concentration occurred was at 20 cm, so all results are given as a percentage of the cell concentration at that level. The results presented in Figure 5.3 illustrate that algal depletion was more evident at the lower current velocities, specially at 0.6 and 3 cm. s^{-1}, and at the lower levels of the water column. Cell concentration profiles at the three lower speeds are all significantly different from the controls (t-test, $P < 0.01$, 0.05 and 0.01 respectively). At higher current velocities the cell concentration profiles are not significantly different from the control profiles, relating to the lower clearance rates observed and the greater vertical mixing.

At the lowest current velocity measured (0.6 cm. s^{-1}) the algal cell depletion was greatest at 10 cm above the sediment surface (Figure 5.3) but this was less pronounced at 3 cm. s^{-1} and had disappeared by 17 cm. s^{-1}.

Algal cell depletion at 10 cm and at 5cm at 0.6 and 3 cm. s^{-1} is significantly different (t-test, $P < 0.01$ for 10 cm at 0.6 cm. s^{-1} and $P <0.05$ for the others) from the cell concentrations at 15 cm. At higher current velocities differences in cell concentrations at 5 cm and 15 cm are all non significant.

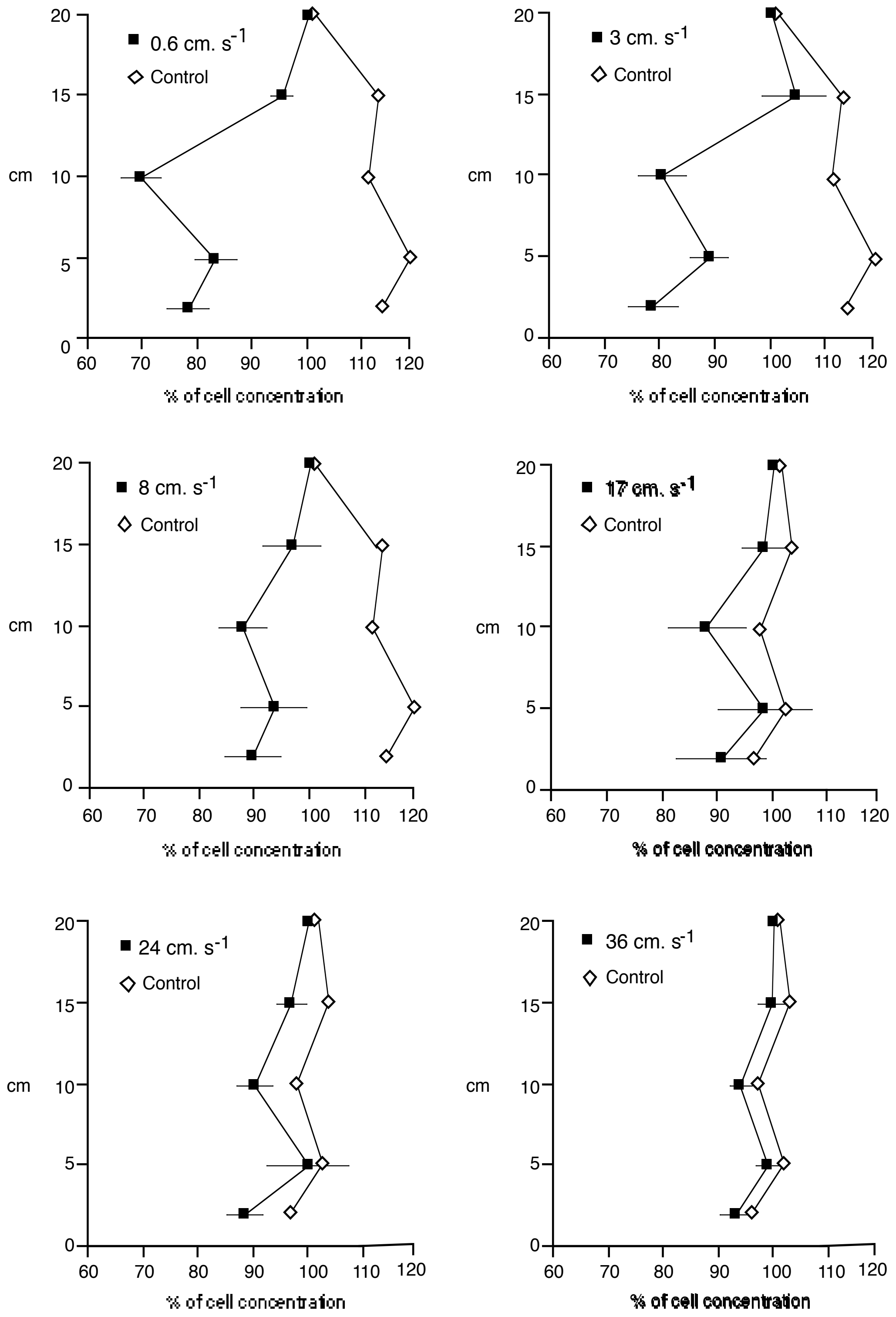

Fig. 5.3 - Vertical profiles of algal cell concentrations (mean ± SE) after depletion by *Ruditapes decussatus* (■) at different current velocities (cm. s^{-1}) in relation to control algal cell concentrations (◊). Results are expressed as % of the cell concentration at 20 cm height.

Algal cell concentrations found at 10 cm are significantly different (t-test, $P < 0.05$) from the cell concentrations found higher (15 and 20 cm) in the water column at all current velocities except at 17 cm. s^{-1} where differences where not significant due to higher variability as shown by the SE (Figure 5.3).

5.3.2. Turbidity and particle size selection

There is a marked decline in clearance rates of clams with increasing seston concentration (Figure 5.4). This decline shows a significant linear relationship for the range tested and is fitted by the equation $y = -0.003x + 1.426$, with $r = 0.99$ ($n = 3$).

At the two higher concentrations visual monitoring was not possible due to the high turbidity but at the end of the experiment the presence of mucus containing some of the particles was observed on the foot, mantle edge and on the bottom of the beakers .

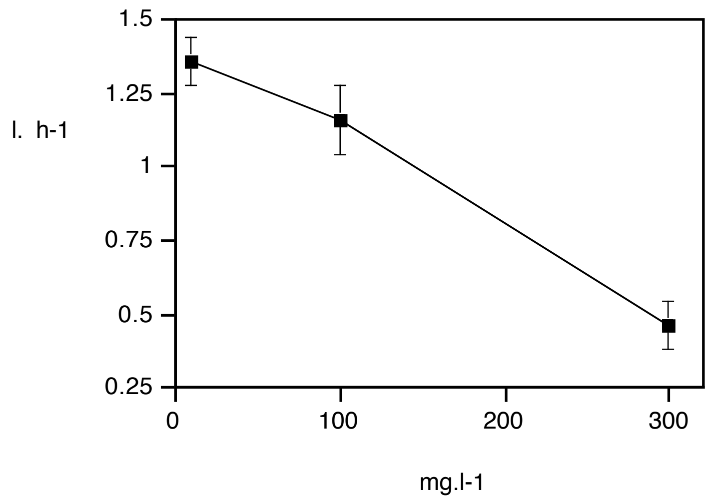

Fig. 5.4 - Influence of turbidity (mg. l^{-1}) on the clearance rate ($l. h^{-1} \pm SE$) of *Ruditapes decussatus.*

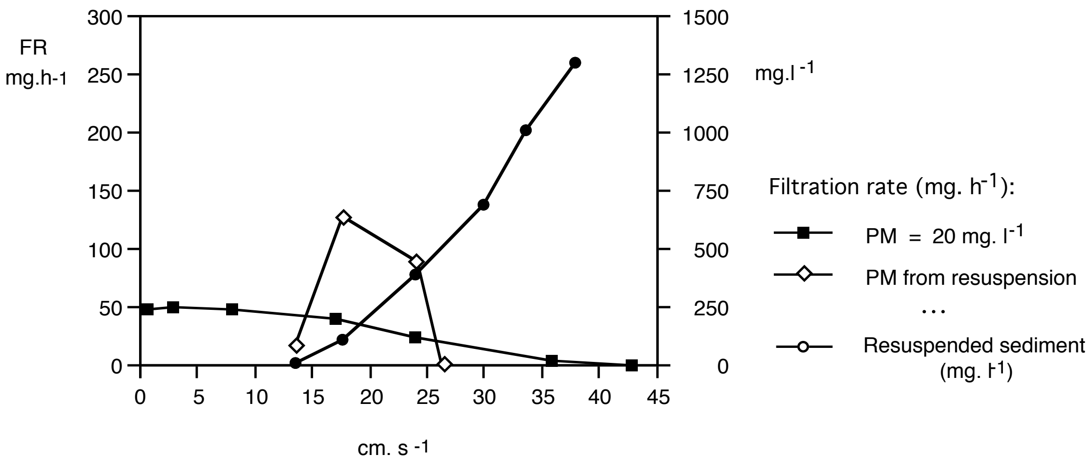

Fig. 5.5 - Relationship between filtration rate (FR, mg. h^{-1}) of *Ruditapes decussatus* and free stream velocity for two seston concentrations (PM, mg. l^{-1}): a fixed concentration of 20 mg. l^{-1} and the concentration of particulate matter resulting from resuspension of sediment with increasing current velocity.

Based on the results from clearance rates at different current velocities (Figure 5.2), filtration rates were calculated for the average turbidity (*c.* 20 mg. l^{-1}) monitored at the sediment surface by an OBS probe at the field sampling site in 1995 (Sobral & Widdows, unpublished) (Figure 5.5). The regression line obtained for the relationship between clearance rates and current velocities was used to calculate the current velocity inhibiting feeding.

It is clear from Figure 5.5 that there is a decline in filtration rate (mg. h^{-1}) at free stream velocities > 17 cm. s^{-1} when resuspension of sediment increases, while at slow current velocities up to 8 cm. s^{-1} filtration is high and rather constant. At a constant seston load feeding is inhibited by current velocity at *c.* 43 cm. s^{-1} .

With increasing current velocities filtration rate increases with resuspension up to *c.* 100 mg. l^{-1} and then declines with increasing seston concentration due to the lower clearance rates caused by loading of the gills with particles. Feeding is inhibited by turbidity at *c.* 480 mg. l^{-1} corresponding to the seston resuspended at a current velocity of *c.* 27 cm. s^{-1}.

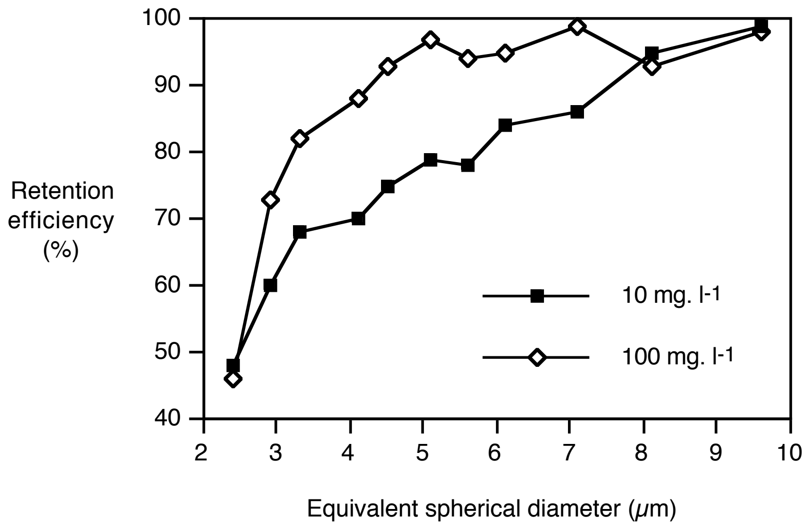

Fig. 5.6 - Particle retention efficiency (%) of *Ruditapes decussatus* under two different turbidity conditions.

The size selection efficiency is different for the two seston concentrations tested (Figure 5.6). The 100 mg. l^{-1} curve shows the normal relationship, with maximum retention efficiency occurring at 4-5 μm. Surprisingly retention of smaller particles (<8 μm) was less effective at the lower seston concentrations. The more effective retention of particles in the range 3-7 μm is higher at 100 mg. l^{-1} compared to 10 mg. l^{-1}, maybe related to extra mucus production at the higher seston concentration and this enhances trapping of smaller particles. This would be particularly beneficial at the higher seston concentration where the algal cells (*c.* 5 μm diameter) were diluted by the fine inorganic silt particles.

Figure 5.7 shows that particles smaller than 3 µm are not retained efficiently (i.e. < 60-70 % retention), and that effectiveness increases with increasing particle diameter. Algal cells (diameter *c.* 5 µm) were filtered with *c.* 80-100 % efficiency.

5.4. Discussion

5.4.1. Current velocity

Provided a wide range of current velocities is tested, the effect of increasing current velocity on bivalve feeding leads to decreasing clearance rates and ultimately to the inhibition of feeding, as has been found for the mussel *M. edulis* (Wildish & Miyares, 1990), the giant scallop *Placopecten magellanicus* (Wildish *et al.*, 1987, 1992) and the bay scallop *Argopecten irradians* (Kirby-Smith, 1972; Cahalan *et al.*, 1989). Grizzle *et al.* (1994) found a positive correlation between flow speed and growth in *Mercenaria mercenaria* for speeds of 2-8 cm. s^{-1} with maximum growth at 2 - 4 cm. s^{-1} and decreased growth at speeds as low as 1 cm. s^{-1} for *Crassostrea virginica*.

It is clear from our results that current velocity has a marked effect on the clearance rates of *R. decussatus* and that this species follows the general pattern of response. The slower currents (≤ 8 cm. s^{-1}) seem to support the highest clearance rates (Figure 5.2) which is in agreement with field observations of clams living in enclosed sheltered areas, on the smooth slope banks of the intertidal where currents are not strong (i.e. < 12 cm. s^{-1}, which appears to be near the threshold for erosion of sandy sediments). As the clams live buried with their siphons open at sediment level, any currents that can resuspend large sediment particles (i.e. sand grains) into the siphon openings are likely to have an inhibitory effect on their feeding rate.

Widdows (pers. comm.) found that *M. edulis,* attached to hard substratum, was able to maintain high clearance rates up to a shear stress of about 3.3 Pa and free stream velocities of *c.* 60 cm. s^{-1}. As *M. edulis* is usually attached to hard substrata and does not usually experience the bombardment by large resuspended sediment particles (i.e. sand grains) and mobile substratum it is not surprising that they are able to maintain clearance rate at higher current velocities.

In contrast, the performance of *R. decussatus* is affected by shear stresses as low as 0.4 Pa, when sediment starts to move, first by the rolling of particles against the siphons, and then by hitting them strongly as saltation and suspension of particles increase with current velocity. The reduction of the siphons opening is the immediate response to these physical stimuli, thus causing clearance rate to decline and even cease by periodic valve closure. The clams were able to cope with the mobile sediment by maintaining their position at the sediment surface and maintaining some pumping activity. At 36 cm. s^{-1}, the highest current velocity tested, clearance rate is reduced to *c.* 10 % of the maximum value, still meeting the oxygen requirements, which are estimated to be c. 0.3 % of the oxygen made available by ventilation rates in normoxia (see Chapter 3).

Algal cell depletion in the water column is apparent at low current velocities (Figure 5.3). Control runs show evidence of less effective vertical mixing at low speeds accounting for most of the algal cells being below the 15 cm level, which was the level at which they were introduced in the flume. However, the vertical profiles shows that the algal cell concentration is relatively constant below 15 cm. At high speeds (17 cm. s-1) algal cells appear evenly distributed in the water column of control runs, without clams.

Introducing the clams has a marked effect on the vertical profile of algal cell concentration particularly at speeds < *c.* 8 cm. s-1.

At the 0.6 cm. s^{-1} free stream velocity, where vertical mixing and turbulence is the lowest, there is a significant difference between 15 and 10 cm and less significant between 15 and 5 cm. In fact the 10 cm level has a cell concentration that is consistently lower than concentrations found at higher levels of the water column at all the current speeds tested, even if this effect is less marked at higher velocities with consequently higher vertical mixing and turbulence. Differences between algal cell concentrations at 5 cm and at 15 cm are not significant for current velocities ≥ 17 cm. s^{-1}.

This lower algal concentration at the 10 cm level is likely to be caused by the exhalant current jetting out water depleted in algal cells at a level between 5 and 10 cm high. André *et al.* (1993) describing the flow pattern of *Cerastoderma edule* of similar size, found that jetting can be as high as 7 cm at current velocities of 1 cm. s^{-1}, about the same velocity used in the present study.

The ability of jetting out depleted water at a different level than the one of the inhalant current is likely to be an important adaptation by clams and cockles living in the slow currents of sheltered environments. This appears to be in contrast to mussels, which cause algal cell depletion immediately above the substrate (Widdows, unpublished). Mussels which have both their siphon openings at the same level and do not have a specific orientation may refilter water that has already been depleted of particles and thus lower the filtration rate (mg. h^{-1}). Figure 5.7 illustrates the consequences of seston depletion at low current velocities on the filtration rate by mussels, and the absence of such a decline in filtration rate by *R. decussatus,* due to the inhalant siphon drawing water from a different level than this depleted layer. Mussels are therefore much more dependent on higher current velocities to maintain seston levels and avoid algal cell depletion and refiltering of depleted water. Consequently clams maintain high filtration rates over a wide range of low current velocities (eg. < 10 cm. s^{-1}, Figure 5.7), whereas musssels, living attached to hard substrata, maintain a constant filtration rate over a wide range of higher current velocities.

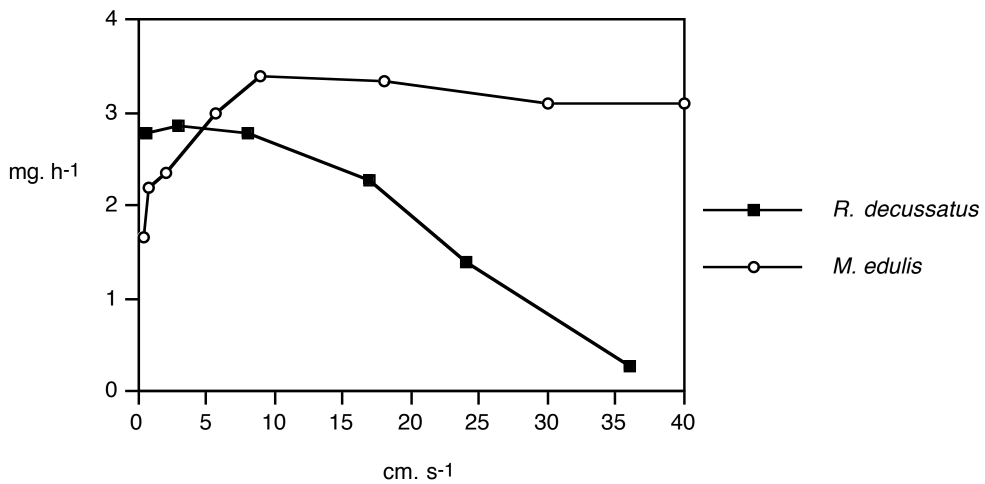

Fig. 5.7 - Filtration rates of *Ruditapes decussatus* and *Mytilus edulis* (mg. h^{-1}. ind^{-1}) for different current velocities at the same algal cell concentration (*c.* 1 mg. l^{-1}).

R. decussatus, being an infaunal bivalve, appears to prefer slow water currents (≤ 8 cm. s^{-1}) that do not generate sediment movement and thus do not disturb their feeding behavior by bombarding the siphons with particles. It is estimated that at 43 cm. s^{-1} feeding rate will be totally inhibited by valve closure. Producing an exhalant current that jets as high as 10 cm above the substrate enables these clams to better exploit their environment, ensuring that the water is pumped in without particle dilution caused by the depleted exhalant current. It is probable that this strategy is common to most infaunal suspension feeding bivalves living in areas with low current velocities.

Scope for growth values calculated for the two species at a free stream velocity of *c.* 0.6 cm. s^{-1} are shown in Table 5.1. These values were calculated using food ingestion rates based on an algal ration of *c.* 1 mg. l^{-1} and different algal depletion profiles (100 %). Mussels have been shown to pump and filter water that is depleted by 25 % at the substrate level (Widdows, unpublished), whereas clams pump and filter water depleted by 22 % (Figure 5.3). However if clams ejected depleted water at the sediment surface rather than at 10 cm height the exptrapolated cell depletion would be 45 %. Respiration rates and absorption efficiencies were taken from the literature (Widdows (1988), Sobral, unpublished) standardized to a 0.3 g dw animal. Values in Table 5.2 show that there is a reduction of *c.* 43 % in the scope for growth of clams ingesting at the same level of the exhalant current compared to clams that ingest at substrate level and exhale at a higher level of the water column confirming the advantages of this strategy. Scope for growth of *M. edulis* shows *c.* 27 % reduction when compared to *R. decussatus* which suggests that the clam is better adapted to grow in sheltered environments.

Table 5.2 - Comparison between energy budgets and scope for growth (J. h^{-1}) of *Ruditapes decussatus* with: (1) water intake and output at different levels (natural situation) and (2) water intake and output at the same level, and (3) *Mytilus edulis* (0.3 g dw) water intake and output at same level (natural situation) at a current velocity of *c.* 0.6 cm. s^{-1} and a 100 % algal ration of 1 mg. l^{-1}. CR - clearance rate C - energy consumed, AE - absorption efficiency, A - energy gain, R - energy loss through respiration, SFG - scope for growth.

Species	**CR** l. h^{-1}	**C** J. h^{-1}	**AE** %	**A** J. h^{-1}	**R** J. h^{-1}	**SFG** J. h^{-1}
R. decussatus (1) intake and output at different levels	2.4	43.1	43	18.5	3.6	14.9
R. decussatus (2) intake and output at same level	2.4	30.4	43	12.1	3.6	8.5
M. edulis	1.8	32.5	40	13.0	2.1	10.9

5.4.2. Turbidity and particle size selection

This study shows that the clearance rates of *R. decussatus* decline with increasing turbidity (Figure 5.4). These findings are in agreement with the observations that high seston concentrations generally reduce the pumping rate as shown by Foster-Smith (1975) for *M. edulis*, *C. edule* and *Venerupis pullastra*, filtering purely algal suspensions, Widdows *et al.* (1979) for *M. edulis* feeding on resuspended fine mud, Grant *et al.* (1990) for *O. edulis,* Grant & Thorpe (1991) for *Mya arenaria,* Iglesias *et al.*

(1992) for *C. edule* all feeding on mixtures of algal cells and suspended silt.

At high seston loads the gills become overloaded with particles and the mechanisms of gill cleaning may account for a significant energy loss (Jørgensen, 1981). This author observed in *M. edulis* that mucociliary mechanisms serve to clean the gills and other organs of the mantle cavity from excess particulate material. This was in fact observed in *R. decussatus* when filtering at the highest seston concentration tested (300 mg. l^{-1}). While production of mucus is basically part of the normal feeding process, excessive production in response to high seston levels may be detrimental and is accompanied by a low clearance rate (i.e. < 0.5 l. h^{-1}).. These observations indicate that high seston concentrations are capable of lowering performance of clams.

Suspended silt at low concentrations added to algal cell suspensions, and thus resembling natural assemblages of seston, has been found to enhance the clearance rates of *M. edulis* (Kiørboe *et al.*, 1980, 1981; Bayne, *et al.* 1987) and *V. corrugatus* (Stenton-Dozey & Brown, 1994). In the present experiments, however, when *R. decussatus* was fed at the lowest resuspended silt concentration (10 mg. l^{-1}) rather than higher the clearance rate of *R. decussatus* was lower than when feeding on algal cells only.

It has been suggested by Møhlenberg & Riisgård (1979), that the absence of sediment may represent a stress and lower the clearance rates of infaunal bivalves. In fact when redrawing Figure 5.4, we observe that for a seston concentration of *c.* 1 mg. l^{-1} (approximately the dry weight of the algal cells ration) the extrapolated clearance rate is *c.* 2 l. h^{-1} (Figure 5.8), a value close to the rates recorded when measuring clearance rate from algal cell suspensions in beakers without sediment, (i.e. similar conditions to those used when examining the influence of hypoxia and temperature on the physiological performance of *R. decussatus,* see Chapter 3 and 4). When the clams are buried in sediment, thus approaching environmental conditions, clearance rates may be higher (Figure 5.8).

An initial study, however, showed that there were no significant differences between clearance rates of clams held in or out of sediment and measured in beakers. The difference found here is therefore probably due to the longer settlement time allowed in the flume experiments (overnight) compared to the shorter settlement time in the beaker experiments (*c*. 2 hours).

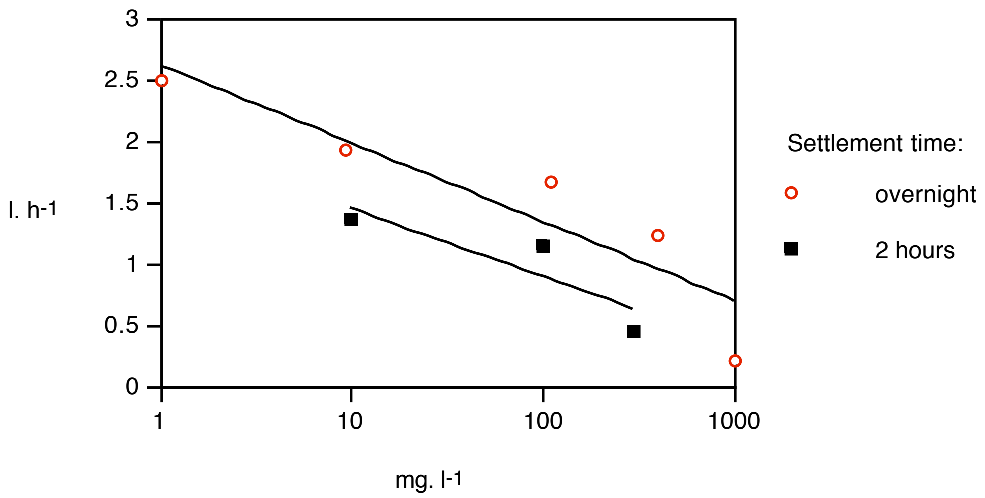

Fig. 5.8 - Influence of seston concentration on the clearance rate of *Ruditapes decussatus*, measured after 2 hours of settlement time, showing the decreased clearance rate at algal food concentrations (*c.* 1 mg. l^{-1}) compared to flume experiments where clams were allowed to bury overnight.

Settlement time thus seems to influence the clearance rate of *R. decussatus*, (reducing it by *c.* 25 % at seston concentrations of *c.* 1 mg. l^{-1}) Consequently this should probably be taken in consideration when extrapolating experimental results to field conditions.

Based on the average turbidity monitored at the sediment surface at the Faro sampling site in 1995, the effect of increasing current velocities on clearance rates combined with turbidity (Figure 5.5) shows that filtration rates are greatly depressed above seston concentrations of *c.*. 100 mg. l^{-1}, approaching zero at seston concentrations of *c.*. 480 mg. l^{-1}. *R. decussatus* appears to control ingestion at high seston concentrations by reducing the amount filtered, lowering clearance rates instead of rejecting particles by producing pseudofeces as has been observed in *Ruditapes philippinarum* by Deslous-Paoli *et al.* (1986).

The pattern of particle retention efficiency shown by *R. decussatus* at the higher seston concentration (100 mg. l^{-1}) ensuring effective retention of particles in the range 3 - 7 µm is particularly beneficial when algal cells (c. 5 µm diameter) are diluted by the fine inorganic silt particles (Figure 5.6). Palmer & Williams (1980) also found evidence of adjustment of the retention efficiency of *Argopecten irradians* and *Crassostrea virginica* in response to different seston concentrations. *A. irradians* was found to increase the retention efficiency of smaller particles (< 4 µm) with increasing seston concentrations, by trapping particles through the production of mucus, probably in the same way as *R. decussatus*. On the contrary *C. virginica* is less efficient in retaining small particles with increasing seston concentrations.

Size selection shows that *R. decussatus* cannot efficiently retain particles smaller than 3 µm in diameter (Figure 5.7), which includes bacteria and clay particles. This is in contrast to *M. edulis* which have high retention efficiencies (80 %) for particles of 3 µm and smaller (Vahl, 1972). The same author found efficiencies of more than 90 % in the range of 4 to 8 µm, slightly higher than those found for *R. decussatus*. Algal cells, such as phytoplankton, which play an important role in the nutrition of the clams, and other particles in the range from 3 to 8 µm are efficiently retained (70 to *c.* 100 % retention).

The pattern of retention efficiency for particles of ≥ 4 µm by *R. decussatus* agrees with the results obtained by Møhlenberg & Riisgård (1978) when examining the retention efficiency of 13 bivalve species. In the lower range (particles < 4 µm) some variability in retention efficiency was observed between species, probably reflecting size distribution of seston in the natural environment. *O. edulis* and *C. edule,* common in estuarine areas where fine sediment occurs, both revealed a steep decline in retention efficiency for particles below 3 µm diameter, similar to the relationship found for *R. decussatus*. This may represent an adaptation of the clams to a richer and more turbid environment, in contrast for instance with the open waters in which mussels live and where lower particles concentrations occur.

6. Physiological Responses of *Ruditapes decussatus* to copper contamination

6. Physiological responses of *Ruditapes decussatus* to copper contamination

Part I - Environmental copper levels in the Ria Formosa

6.1. Introduction

6.1.1. Copper in the environment

Copper contamination of estuarine and coastal marine environment (i.e. above a background concentration of 2-5 g Cu. l^{-1}) is directly attributable to industrialization and development. About 81 % of the total production of copper has taken place in this century, and the all time mine production is estimated to be 307 million metric tons (Moore & Ramamoorthy, 1984). Copper, like other heavy metals, has been the subject of increasing research activities to determine and ultimately to control their concentration in estuarine and coastal marine habitats, due to its persistence in the environment, its toxicity at high concentrations, and its tendency to accumulate in the biota with potential hazard to man (Kennish, 1992).

Copper is a widely distributed metal in nature due to its universal use in many industries, such as electrical, construction, plumbing and automotive. The electrical industry accounts for more than 50% of its use in power transmission, generation and consumption of electrical energy, electronics and electrical equipment.

Mine tailings and flyash (major source of solid copper waste) account for 75 % of the copper input to the atmosphere, other anthropogenic sources being fertilizer production, and municipal and industrial sewage. It is estimated that 5000 to 13000 metric tons per year of copper are precipitated from the atmosphere into the oceans (Nriagu, 1979 *in* Moore & Ramamoorthy, 1984).

Waste disposal in estuarine and coastal areas (*c.* 17000 metric tons of copper per year), and dredged-material disposal, associated with solid copper phases accumulate this metal in the sediments making its biological effects more conspicuous among the benthic communities (Kennish, 1992).

The use of copper as a biocide, specially in antifouling paints has decreased considerably during the 80's. In Portugal, the use of copper sulphide ($CuSO_4$) as a biocide against vine pests, a common procedure in the past, is becoming rare, but remaining mine tailings from deactivated copper mines are an important source of copper to the environment. In terms of environmental pollution copper is thus a very toxic and relatively accessible metal.

Copper levels in oceanic waters on the Atlantic Ocean fall in the range 0.1-0.04 $\mu g.\ l^{-1}$ (Wittmann, 1981), but in coastal waters due to impact from point discharges and different water circulation conditions, concentrations show wide variations. Generally a value between 2 and 5 $\mu g.\ l^{-1}$ of copper is accepted for non-polluted coastal areas (Davenport & Redpath, 1984).

Sedimentary copper levels are usually much higher, up to 20 mg. kg^{-1} for unpolluted sediments (Moore & Ramamoorthy, 1984).

Copper levels in marine invertebrates reflect the presence of bioavailable copper that is sequestered in the tissues and are generally < 60 μg. g^{-1} for molluscs and crustaceans of Europe and North America (Moore & Ramamoorthy, 1984).

6.1.2. Effects of copper on marine organisms

Copper, like other metals, can exist in sea water systems in a variety of states depending on physical, geochemical and biological processes (microbial activity, bioturbation and biodeposition) which largely determine their bioavailability and thus their toxicity. Copper speciation in sea water is outside the scope of this work and has been reviewed by Zirino & Yamamoto (1972) and Byrne *et al.* (1988). According to Moore & Ramamoorthy (1984), 40 to 60% of total copper in estuarine and coastal waters is associated with colloidal matter forming organic and inorganic complexes. Ionic forms of copper, tacitly assumed as the most toxic, make up only 1 to 2 % of total copper in sea water (Davenport & Redpath, 1984).

There is a fine line between the requirement and toxicity of copper. Copper in trace quantities is an essential metal for metabolic processes used as a cofactor in proteins and a constituent of enzymes like the cytochrome oxidase and certainly, marine bivalves are obligate accumulators of essential heavy metals (White & Rainbow, 1985). On the other hand the binding of excess copper to specific sites in proteins can disrupt several metabolic processes (Viarengo *et al.*, 1981a), providing a mechanistic basis for its deleterious effect at higher levels of biological organization (Weber *et al.* 1992). Copper is thus a beneficial trace element required by all marine organisms, yet one of the most poisonous of heavy metals when present in excess (Davenport & Redpath, 1984).

Toxicity and accumulation of copper is dependent on speciation and bioavailability and therefore varies with the physical and chemical factors like the presence of chelators, the concentration level, spatial and temporal complexity of the exposure, temperature, salinity, presence of other metals, and with biological factors such as the stage of the life history, size of the animal, reproductive condition and population density.

Though results are difficult to compare due to different methodologies, according to a review by Patin (1982), the lethal copper concentration for 50 % of the individuals of a population is generally less than 1 mg. l^{-1} for marine bivalves, though the time it takes to reach this mortality varies between 2 and 75 days. Reduced copper concentrations (sublethal levels) lead to longer survival times. The toxicity threshold is generally < 0.2 mg. l^{-1}.

Most bivalves accumulate trace metals in direct proportion to their environment levels, storage occurring in metal binding proteins at low contaminant levels or in granules at high levels, and are therefore considered partial regulators of trace metals (Phillips & Rainbow, 1989).

In filter-feeding bivalves the uptake of copper is done via ingestion of particulate matter or directly by diffusion of dissolved forms through the water-tissue contact surfaces. Copper homeostasis is linked to complexation by induced metal binding proteins within the cell solution, like metallothioneins, as it has been shown by Viarengo *et al.* (1980), and Roesijadi (1980, 1981) and subcellular inclusions such as insoluble granules, lysosomes and vesicules (Moore, 1985). Metal binding proteins, that bind essential metals (Cu and Zn) as well as pollutant metals (Cd, Hg and Ag), and inclusions detoxify metals and provide the basis for accumulation (Mason *et al.*, 1984). Toxicity occurs when these compensatory mechanisms are saturated or damaged by the metal influx.

Bivalves accumulate copper conservatively, the gills being the principal target organ (Roesijadi, 1980), where it affects the ciliary feeding activity (Widdows & Donkin, 1991). A suppression of the lateral ciliary action due to destruction of the gill epithelial tissues thus impairing ventilation rate was observed in *Mytilus edulis* at 0.5 mg Cu. l^{-1} by Manley (1983). Reduced respiration rates (50 %) were observed for the same species exposed to *c.* at 200 - 250 μg Cu. l^{-1} (Delhaye & Cornet, 1975). Abel (1976) reported a 50 % reduction on the filtration rate at copper concentrations between 80 - 230 μg Cu. l^{-1}, and Manley (1983) could detect a threshold for disturbance of filtration rate at *c.* 10 μg Cu. l^{-1}. Reduction of byssus thread production has been shown as a response to copper (Davenport, 1977), and the profound effects on protein metabolism in *Mytilus galloprovincialis* were demonstrated by Viarengo *et al.* (1981). Growth being the result of behavioural, physiological and biochemical functions is likely to be specially sensitive to the action of pollutants such as copper (Davenport & Redpath, 1984).

Studies on the effects of copper on the physiological performance of bivalves other than mussels are scarce. Katticaran & Salih (1992), studied these effects on the clam *Sunetta scripta*. Nothing is known about the way *R. decussatus* accumulates copper at environmental levels or responds to toxic (lethal) levels. Physiological disturbances in this species have not been measured under metal stress and the alterations in terms of scope for growth are unknown.

As no data on copper inputs to the Ria Formosa are available and only scarce information on copper concentrations in the Ria was found (Cortesão *et al.*, 1986), a sampling program was designed to measure actual field concentrations at a single clam farm near Faro. General characterization of the field site has already been presented in Chapter 2. Such a program may then enable environmentally realistic concentrations to be studied in accumulation experiments (Part II) performed in the laboratory.

6.2. Materials and methods

6.2.1. Field sampling methods

Copper concentrations in the clams and sediment, sampled from the study site (Figure 1) were measured on a seasonal basis between March 1992 and December 1993. The sampling measurements of copper in water and pore water particulate matter (PM) were carried out between in October 1992 and December 1993.

Water samples were collected from the overlying water of a shallow area of the clam farm. Pore water (sieved through a 200µm mesh), sediment and clams were collected in an area recently exposed by the tide.

All samples were collected in polyethylene containers previously decontaminated for 24 hours in a nitric acid (HNO_3) solution 1:1, thoroughly rinsed with desionised water and dried. After collection they were kept in coolers at about 10-15 °C, during transportation to the laboratory. Clams were transported in a similar cooler in plastic net bags, without water.

6.2.2. Laboratory procedures

Care was taken that all utilized material had been previously decontaminated.

In the laboratory water samples were homogenized and a known volume was filtered through polycarbonate Nuclepore® filters, of 0.4 μm mesh size. The filters were then oven dried at 60 °C during 48h. Water samples were preserved with HNO_3, (2 ml. l^{-1}), and kept refrigerated. Sediment was also oven dried in the same conditions as PM. Clams were measured (antero - posterior length) with calipers, separated into four length classes between 25 mm and 45 mm, frozen for a few hours and allowed to open. The soft parts were washed with 0.5 M ammonium formate to eliminate salt, separated from the shell with a plastic knife and oven dried, in small plastic layered Petri dishes, at 60 °C to constant weight (not more than 48h).

6.2.2.1. Preparation of samples for copper analyses

All copper measurements were made by atomic absorption spectrometry (AAS) with a Instrumentations Laboratory (IL) S11 flame spectrometer.

The working area was kept clean of dust and fumes so as to avoid contamination.

All the labware used in preparing the samples was previously decontaminated in a 1:1 HNO_3 solution. For small volumes transfer pipettes were used. All reagents were analytical grade and conductivity of distilled water was less than 4 $\mu S.cm^{-1}$.

6.2.2.1.1. Water samples

Water samples of 100 ml were prepared by extraction into freon using as complexing agent a solution of ammonium pyrrolidine dithiocarbamate (APDC) and diethylammonium-N,N-diethyldithiocarbaminat (DDDC), and acetate buffer 1:1, in Teflon extraction bottles.

The metals are then back extracted in 100µl of HNO_3 to which distilled water is added to make up a final volume of 5 ml, the initial sample being thus concentrated 20 times. The detailed procedures for the preparation of seawater samples for copper determinations are given in Appendix III.

All samples were prepared in duplicate. For each batch of samples two controls, i.e. distilled water, were used.

Analytical methodology was tested using the certified reference material SLRS - 2 (Riverine Water Reference Material for Trace Metals) from the Marine Analytical Chemical Standards Program of the National Research Council of Canada, and our results were within 23 % of the certified value.

6.2.2.1.2 Sediment samples

Samples of approximately 0.7g dry weight were ground in an agate ball mill and digested with *aqua regia* (HNO_3 and chloridric acid HCl, 1:4) and fluoridrid acid (HF) in closed Teflon vessels at 100 °C in a water bath, for 1h 30 m. Boric acid (HBO_3) and distilled water was added to enhance metal fluoride dissolution and the sample was recuperated in 50 ml volumetric flasks. This extraction method totally dissolved the samples. The detailed procedures for the preparation of sediment samples for copper determinations are given in Appendix IV.

All samples were prepared in duplicate and two controls, i.e. only the reagents, were used. Analytical methodology was tested using the certified reference material BCSS - 1 (Marine Sediment Reference Material for Trace Elements and Other Constituents) from the Institute for Environmental Chemistry of the National Research Council of Canada. Our results were within 16 % of the certified value.

6.2.2.1.3. Particulate matter samples

This technique is similar to the one used for the sediment samples, except for the volume of reagents used and time of digestion. Filters were digested with *aqua regia* and HF in closed Teflon vessels at 100 °C in a water bath, for 1h. HBO_3 and distilled water was added to enhance metal fluoride dissolution and the sample was recuperated in 25 ml volumetric flasks. The detailed procedures for the preparation of particulate matter samples for copper determinations are given in Appendix V.

All samples were prepared in duplicate and two controls, i.e. only the reagents, were used.

6.2.2.1.4. Clam tissue samples

In order to obtain 1g dry weight for the analysis, 5 to 10 clams (soft parts) were pooled and ground in an agate ball mill. The sample was then digested with HNO_3, for 4 hours in closed Teflon vessels at 100 °C, in a water bath. Hydrogen peroxide was then added and digestion continued for another hour. The sample was recuperated in 25 ml volumetric flasks. The detailed procedures for the preparation of clam tissue samples for copper determinations are given in Appendix VI.

All samples were prepared in duplicate and two controls, i.e. only the reagents, were used. Analytical methodology was tested using the certified reference material CRM 278 (mussel tissue) from the Commission of the European Communities. Our results were within 3.3 % of the certified value.

At this stage all samples were kept refrigerated until measured by AAS.

6.3. Results

Copper concentrations in the particulate fraction of the overlying water followed closely the concentrations found in the water (Figure 6.1). Copper in particulate matter (PM) averaged 59 % of total copper in the water.

Mean values are 2.5 ± 1.0 (SE) μg Cu. l^{-1} for the water and 1.5 ± 0.8 μg Cu. l^{-1} for PM.

Copper in PM appears to follow a seasonal cycle with high concentrations in autumn/winter and low concentrations in spring/summer, probably reflecting rainfall flushing Cu into the lagoon or resuspension from bottom due to increased water turbulence induced by winter winds.

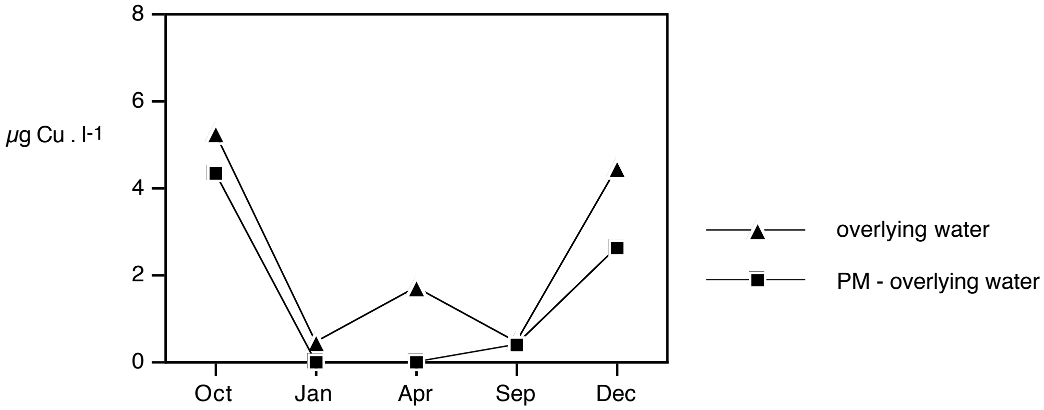

Fig. 6.1 - Total and particulate copper (μg. l^{-1}) seasonal variation in the overlying water at Faro sampling site.

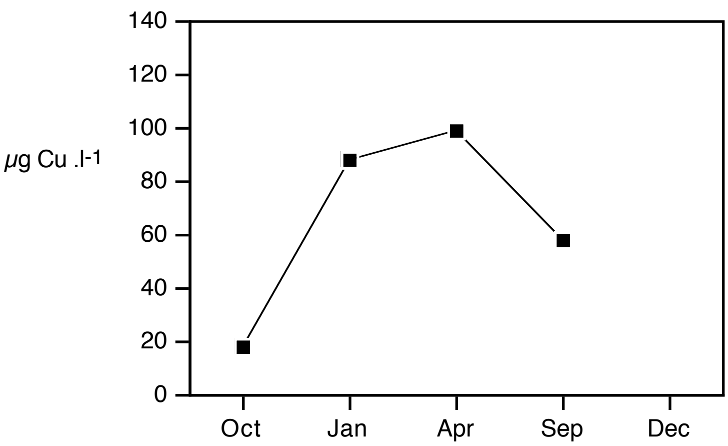

Fig. 6.2 - Seasonal variation of particulate copper (μg. l^{-1}) in pore water at Faro sampling site.

Copper in the particulate fraction of pore water seems to follow the same variation pattern as the concentrations found in the sediment (Figs. 6.2 and 6.3).

Mean copper concentration in the PM of pore water is 66.1 ± 18.2 μg. l^{-1}. Copper in the sediment seems to be stable (mean 53.8 ± 1.7 mg Cu. Kg^{-1}), fluctuations are smaller and similar in both years (Figure 6.3).

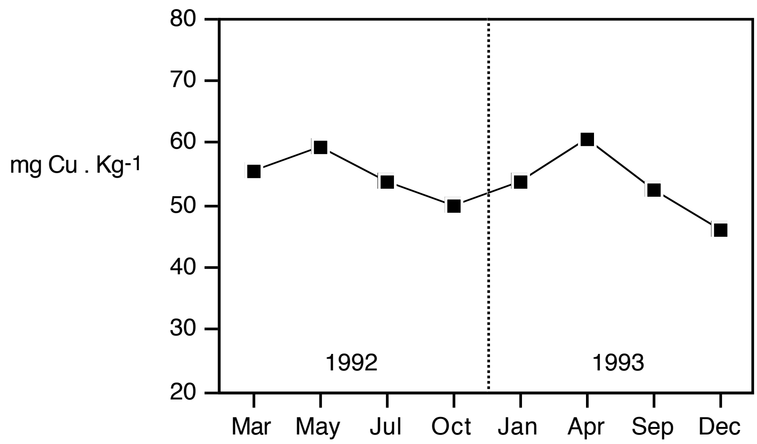

Fig. 6.3 - Seasonal variation of copper (mg. Kg^{-1}) in the sediment at Faro sampling site.

Copper concentrations in the four size classes showed an inverse relationship between animal size and copper concentrations in the tissues. There was a highly significant (one-way ANOVA, $p>0.01$) inverse relationship between body condition index, defined as the ratio dry weight : length of shell, and copper concentration in the tissues (Figure 6.4).

In Figure 6.5 all classes were pooled to show that the seasonal pattern of copper concentration in the clams was similar in the two years, though we can find a higher variation in the second year. Values ranged from 7.0 to 10.8 μg. g^{-1} dry weight, with mean value 8.6 ± 0.4 μg Cu. g^{-1} for the whole sampling period.

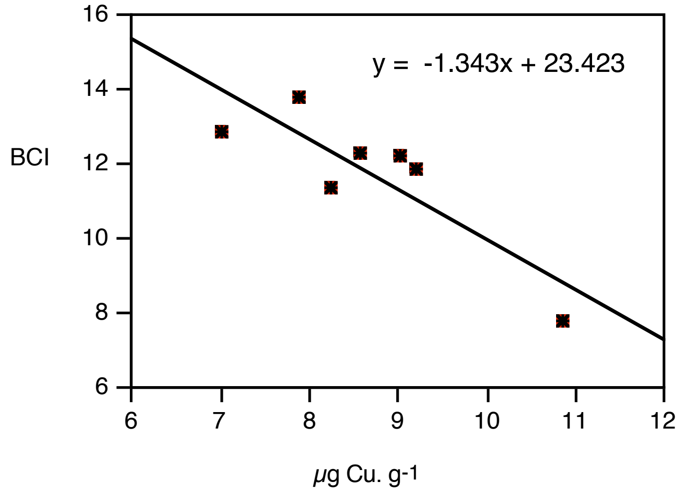

Fig. 6.4 - Fitted regression line, (r= -0.86, n=7), for the relationship between body condition index (BCI) and copper concentration (μg. g^{-1} dw) in the tissues of *Ruditapes decussatus.*

The copper experiments were based on the size class 30 - 35 mm and therefore the seasonal variation in copper concentration of this class is shown in Figure 6.6. The seasonal pattern is very similar to the averaged values of Figure 6.5, (mean of 8.9 ± 0.4 μg. g^{-1}).

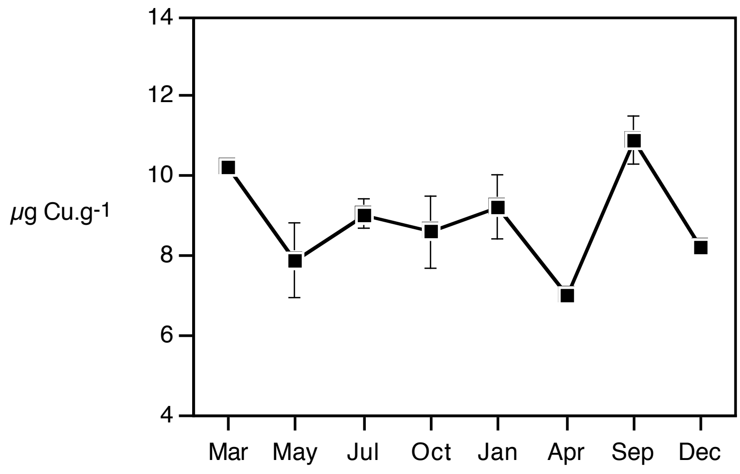

Fig. 6.5 - Seasonal copper concentration (μg. g^{-1} dw ± SE) in soft parts of *Ruditapes decussatus* during the sampling period at Faro site. Results are averages of the four length classes studied.

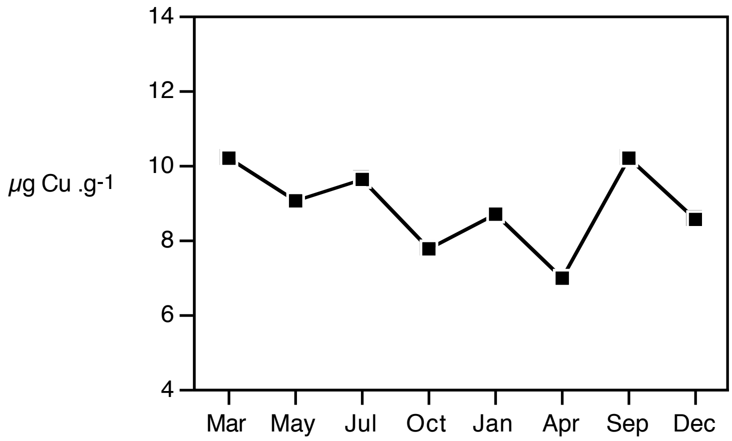

Fig. 6.6 - Seasonal copper concentration (μg. g^{-1} dw) in soft parts of *Ruditapes decussatus* during the sampling period for the length class 30 - 35 mm .

6.4. Discussion

Copper concentrations found in the sea water at the Faro site (mean 2.5 μg Cu. l^{-1}) are comparable to reported data from coastal waters of the Mediterranean and Baltic Seas in the range 1 to 5 μg Cu. l^{-1} (see Moore & Ramamoorthy, 1984) and are half the lowest copper levels reported from Tenerife, Canary Islands (Díaz *et al.*, 1990).

The sediment copper levels (mean *c.* 54 mg Cu. kg^{-1}) agree with the results found by Cortesão *et al.* (1986) for the same area, and are similar to the levels found in the Humber and Severn estuaries, UK., (Bryan & Langston, 1992). They are generally lower than those reported for anthropogenically impacted areas like Cardiff Bay, UK (Hitchcock & Thomas, 1992) and the Bay of Biscay, northern Spain (Legórburu &

Cantón, 1992). They are however higher than the values generally accepted for non-polluted sediments, < 20 mg Cu. kg^{-1} (Moore & Ramamoorthy, 1984).

The results of this study confirm that the Ria Formosa is not subjected to significant copper pollution. In fact it is not an industrialized area although non quantified anthropogenic inputs from the nearby city of Faro contribute to raised copper levels especially in the sediments.

There are two major patterns of seasonal variation in the copper concentrations. The overlying water is characterized by low spring/summer values and high autumn/winter values, related probably to higher run-off due to rainfall in autumn/winter. In contrast the sediment and PM of pore water shows an inverse pattern, low values in autumn/winter and high values in spring/summer. This pattern may be associated with the higher amounts of organic matter, to which copper can be adsorbed, that are normally found in spring and summer (see Chapter 2), due to higher temperatures enhancing decay of detritus and creating reduced conditions near the sediment surface. In fact, the redox potential (Eh) of the sediment was generally negative even at 1 cm depth, indicating no oxygen penetration to that depth (see Chapter 2, Figure 7).

The physicochemical conditions of the overlying and pore water determine processes such as sorption/desorption, precipitation/ solubilization, flocculation and complexation, that largely determine the mobilization of metals and their bioavailability. This change in redox conditions can release part of the heavy metal load sorbed to the sediments, and thus increase metal concentrations in pore water (Förstner, 1981).

Being a burrowing siphonate bivalve *R. decussatus* pumps water at sediment level and so the overlying and pore water (specially the particulate fraction), and the sediment are relevant to the uptake and accumulation of copper by the clams. Organic particulates contribute in a significant way to heavy metal geochemistry influencing flocculation and the mineralization rate in the sediment (Arjonilla *et al.*, 1994), so that when organic matter is involved we can expect mobilization of copper from sediment and subsequent accumulation in benthic bivalves.

Bivalves have been commonly used as indicators of metal pollution due to their ability to accumulate trace metals in direct proportion to environment levels. Significant correlations between metal content and environmental exposure are not always found, due to the extreme variability of physical and geochemical conditions that determine bioavailability of metals and to the physiological/reproductive state of the individuals. Amiard *et al.* (1986), did not find any correlation between metal concentrations in the mussel *M. edulis* and dissolved and particulate levels in the water. Cain & Luoma (1990), found a strong correlation between metal content in *Macoma balthica* and sediment concentrations, but in this study no significant correlation between copper levels was found.

Copper concentrations in *R. decussatus* from the Ria Formosa (mean 8.4 μg Cu. g^{-1} dw) agree with results from Cortesão et al. (1986) and Ferreira *et al.* (1989) in the Ria and are slightly lower than those found by Stenner & Nickless (1975) in Faro and Perdicaro (1985) in the Venice lagoon.

These results are however higher than those reported by López-Artíguez *et al.* (1989) for clams from the Huelva estuary, southern Spain, a heavily polluted area.

Copper concentrations in the clams are in the same range of results from *Cerastoderma edule* (Segar *et al. in* White & Rainbow, 1985), *Cerastoderma glaucum* (Arjonilla *et al.*, 1994) and *M. edulis* (Stenner & Nickless 1975, Boalch *et al.*, 1981, Simkiss *et al.*, 1982, Coimbra & Carraça, 1990) not exposed to significant copper pollution.

The seasonal pattern observed, with low values in spring when clams show their maximum weight, i. e., before spawning, and high values in summer, i. e. after spawning, also suggested by Cortesão *et al.* (1986) can be simply a weight effect related to the physiological processes involved. Seasonal variations in the weight of different tissues are known to influence the relation between metal content and body size (Boyden, 1977). In autumn-winter when the animals are building up their reserves, a new decrease in copper concentrations occurs. The significant inverse relationship found between body condition index and copper concentration in the tissues of *R. decussatus* seems to illustrate this.

According to White & Rainbow (1985), metabolically functional copper can contribute significantly (3 - 50 $\mu g. g^{-1}$) to the metal content of molluscs without hemocyanin as a respiratory pigment, collected from non-polluted environments. Therefore it appears that the observed seasonal cycle in tissue copper concentration of *R. decussatus* is simply reflecting natural fluctuations in the reproductive/physiological conditions of the clams.

Part II - Ecotoxicology. Copper toxicity and its influence on the scope for growth of the clam *Ruditapes decussatus.*

6.5. Ecotoxicology

Ecotoxicology is concerned with protecting ecological systems from adverse effects by chemicals, anticipating the effects and assessing the changes in ecological systems (Calow, 1993). The ultimate objective of ecotoxicological studies is both to predict and diagnose the causes of biological / ecological effects resulting from exposure to chemicals and other stressors in the environment. Therefore the establishment of cause-effect relationships between contaminant concentration and the resultant biological effects, based on their mode of toxic action, is needed.

The responses of organisms to toxic chemicals can be manifested at four levels of biological organization: biochemical and cellular responses, organismal, including the integration of physiological, biochemical and behavioural responses, population, including alterations in population dynamics, and community, resulting in community structure and dynamics.

Experimental studies directed at determining effects on energy metabolism or influence on growth and reproduction would be most appropriate for linking effects to higher levels of organization (Capuzzo *et al.* 1988). Physiological responses to metals are generally non-specific and separating metals effects from other factors can be quite difficult, resulting in ambiguously relating a detected change with biotic and abiotic confounding variables rather than with metal exposure (Luoma & Carter, 1991)

Due to the diversity of environmental situations the combination of the analysis of contaminant levels in the tissues and the measurement of biological effects in terms of physiological energetics is ideally suited to assess this cause-effect relationship (Widdows & Donkin, 1991, 1992)

There are two major approaches in ecotoxicological research. One involves acute lethal tests, dealing with short term exposures with generally irreversible effects, resulting in death or impairment of swimming capacity, or another easily detectable malfunction. Another approach involves chronic long term exposures where bioaccumulation and different types of sub-lethal biological responses can be measured at the organismal (physiological responses) or suborganismal level (biomarkers, i.e. biochemical or cellular responses). Sublethal effects are capable of providing early warning of adverse effects at population and community levels and are more relevant to environmental levels of contamination.

Marine and estuarine species commonly used for testing toxicity and aquatic environmental quality include rotifers, crustaceans and bivalves, selected on the basis of availability at low cost, ease of handling in the laboratory, past sucessful use and commercial and ecological importance (Widdows, 1993).

Bivalves, and particularly the mussel *M. edulis* has been extensively used as an "indicator" organism in marine environmental monitoring programmes throughout the world. In fact, bivalves are dominant members of coastal communities, are sedentary and therefore integrators of contamination in a given area, are relatively tolerant of a wide range of environmental conditions, are able to filter large quantities of water and particulates and concentrate chemicals in their tissues by factors of 10 to 10^5 relative to the concentration in the water, they can be transplanted and are a commercially important seafood species and therefore its contamination is of interest for public health considerations.

6.5.1.Acute exposure

6.5.1.1. Introduction

The need to regulate the introduction of chemicals in the environment led to the development of several acute exposure toxicity tests, that have been standardized and are used routinely in many countries to predict acceptable concentrations in the environment.

These tests have been devised according to several performance criteria: ecological relevance, to predict precise ecological effects or at least to allow no or minimum effect levels to be defined; reproducibility, in order to enable standardization; reliability and robustness, to make possible to carry out tests on demand and in routine procedures; and repeatability/sensitivity to ensure good replicate responses and allow credible distinctions between treatments.

Inevitably, most of the time it is difficult to meet all these criteria simultaneously and compromises have to be made, leading to simplification of the experimental systems which conflicts with the complexity associated with most natural ecological systems (Calow, 1993).

Many of the aquatic acute tests that have been developed rely mainly on the lethal response of a batch of animals to a certain concentration of a toxicant. Mortality is therefore the classical parameter in ecotoxicological research, which is known as the LC_{50}, i. e., the lethal concentration for 50% of the test animals during a defined exposure time. This irreversible effect is measured as an acute response, which means that it has to be effective in a short period of time, typically 96h.

Acute biological tests are defined as static and so suffer from important constraints: they cannot be run for long periods as dissolved oxygen can drop to critical levels, products from excretion can accumulate and also have a toxic effect, and the majority of toxicants (metals and organics) accumulated in the tissues will not be near steady state with the concentration in the seawater.

This form of bioassay is performed in two steps, the first one being the preliminary assay in which a wide range of concentrations is tested and the second one, the definitive assay, in which a finer range is investigated. This finer range is identified in the first step as the interval between the concentrations where no mortality and 100 % mortality occurs.

Our purpose is to investigate the behaviour of *R. decussatus* acutely exposed to copper and to establish the 96h - LC_{50}.

6.5.1.2. Materials and methods

Clams were collected in March 1994 at our sampling site in Faro and allowed to acclimate to laboratory conditions. Temperature was 15 °C and salinity 35.6 ‰ . All tested clams belonged to the size range 3.0 - 3.5 cm (shell lenght).

Nominal copper concentrations were 0.1, 0.5, 1, 5 and 10 mg. l^{-1}, made up from a stock solution of 1g. l^{-1} of copper chloride. Water was aerated to full saturation prior to the experiment.

Two liter plastic containers with five clams in each were used. The test was run in duplicate, with two controls, for 5 days in static conditions with no aeration. The animals were not fed during the experiment.

Observations were registered daily. Dissolved oxygen was never below 60% of saturation.

6.5.1.3. Results

Only two animals died in the whole experiment, one in the 5 mg Cu. l^{-1} concentration on the third day and another in the 0.5 mg Cu. l^{-1} concentration on the fourth day. No mortality occurred in the controls.

Data were therefore insufficient to proceed to the definitive step and it was not possible to calculate the 96h - LC 50.

Feces were observed only in the two lowest copper concentrations (0.1 and 0.5 mg Cu. l^{-1}) and in the controls. Production of mucus (as white aggregates) was observed since the first day, in concentrations 1, 5 and 10 mg Cu. l^{-1}.

In the two higher concentrations a bluish film resulting from copper precipitation, covered the bottom of the containers as well as the clams. These clams remained closed for most of the duration of the experiment and they were found to be alive when returned to normal sea water.

6.5.1.4. Discussion

The presence of feces in the two lowest concentrations (0.1 and 0.5 mg Cu. l^{-1}), indicates that clams have opened their valves and pumped water. At 1 mg Cu. l^{-1} and at higher concentrations, mucus production is indicative of stress, and suggests that the 96h LC 50 will probably have a value between 0.5 and 1 mg Cu .l^{-1}, if the animals were pumping continuously. Closing the valves is an evasive mechanism that enables *R. decussatus* to survive short-term exposure to high toxicant concentrations. Scott & Major (1972) reported copious mucus production in *M. edulis* exposed to 0.3 mg Cu. l^{-1} and complete closing of the valves at 10 mg Cu. l^{-1}.

Clearly, the acute ecotoxicological test is not suitable for animals such as bivalves that can remain closed for long periods (over five days) and so escape in the short term the exposure to toxicants in seawater. Of course, it can be assumed that during prolonged chronic exposure, the clams would accumulate toxic anaerobic end products and deplete their reserves and eventually resume pumping. Only at this stage would they become intoxicated and mortality would be apparent.

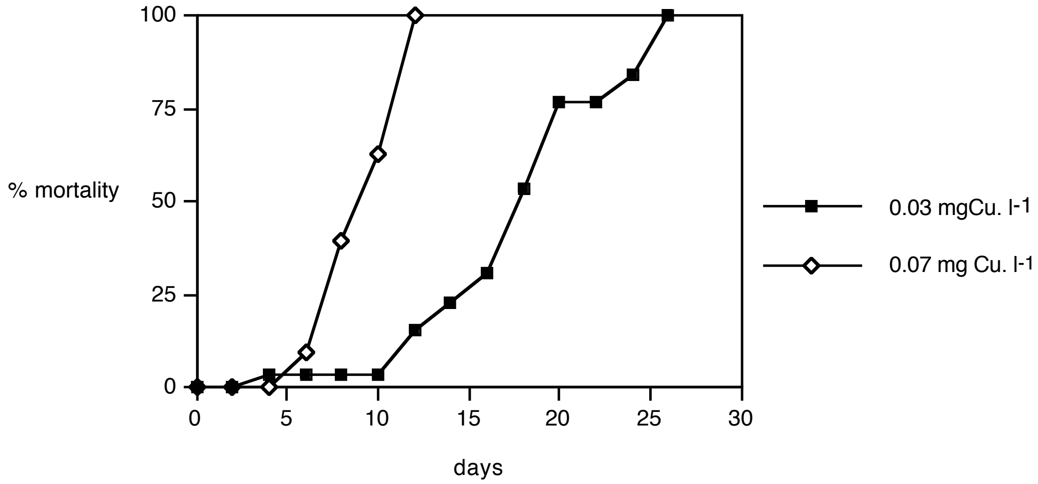

Fig. 6.7 - Mortality of *Ruditapes decussatus* exposed to 0.03 and 0.07 mg Cu. l^{-1} in an accumulation experiment (adapted from Sobral *et al.* 1992).

In a previous accumulation study (Figure 6.7) at 15 °C, Sobral *et al.* (1992), found that 0.07 mg Cu. l^{-1} killed all the clams in the experiment in 12 days (50% in *c.* 8 days) and 0.03 mg Cu. l^{-1} killed 100 % in 26 days (50 % in 18 days). Data from Stephenson & Taylor (1975) for *R. decussatus* exposed to copper at the same temperature as in our experiments, indicate that 0.01 mg Cu. l^{-1} causes 50 % mortality in 75 days and 0.1 mg Cu. l^{-1} in *c.* 35 days. It is obvious that exposure times much longer than those generally accepted for acute (short-term) tests are needed to establish the LC_{50} for this species.

Acute tests, defined as short term tests that have been successfully applied to rotifers, crustaceans and even bivalve larvae (reviewed by Widdows, 1993) are therefore not applicable to adult clams of this species or the majority of adult bivalves (Widdows & Donkin, 1992).

6.5.2.Chronic exposure

6.5.2.1. Introduction

Since many pollution situations involve long term exposures at low concentrations the chronic exposure approach is more realistic than the acute tests regarding the conditions of natural ecological systems. These type of experiments measure accumulation of a toxicant and also include sub-lethal responses to its accumulation. The cause-effect relationship is best studied by the combination of chemical analyses of the contaminant levels in the body tissues and the measurement of biological effects in terms of physiological energetics (Widdows & Donkin, 1991, 1992).

Very few standard methods have been developed for measuring bioaccumulation, especially in marine organisms (Phillips, 1993). Generally the experiments are run for long periods of time, weeks or months, and the concentrations used are always sub-lethal. These tests are performed in semi-static or preferably in flow-through conditions. In semi-static conditions the media is renewed periodically, generally every day or every other day.

Sub-lethal physiological responses are particularly useful in assessing the biological effects of pollution on the growth potential of an organism.

The rate of growth is one of the most sensitive measures of stress in an organism and part of the difficulty of quantifying and interpreting it can be overcome by the determination of the energy available for growth, or scope for growth (SFG).

The physiological analyses of the energy budget, on which SFG is based, not only reflects the integrated toxic effects of accumulated toxicants but also provides insight into the underlying mechanisms of toxicity and the components which effect changes in the growth rate (Widdows & Donkin, 1992, Widdows, 1993).

The objective of this experimental study is to investigate the behaviour of the clam *Ruditapes decussatus* exposed to a chronic level of copper contamination and show the sensitivity of the physiological approach to detect deviations from normal performance at environmental copper levels.

As described in Chapter 2, the individual physiological responses, i. e., clearance rate, respiration rate and absorption efficiency are converted into energy equivalents and used in the balanced energy equation of Winberg (1960) to calculate scope for growth.

6.5.2.2. Materials and methods

Clams (32.5 mm shell length ± 0.32, n = 128) were collected at low tide in April 1994 from the sampling site in Faro and allowed to acclimate to laboratory conditions (temperature 20 ± 1 °C, salinity 35.6 ‰) for 4 days.

Thirty-five clams were held in plastic tanks containing 10 l of seawater.

Clams were exposed to a copper concentration of 0.01 mg. l^{-1}, obtained by diluting a stock solution of 1g. l^{-1} $CuCl_2$. There were two copper exposure tanks and two control tanks with 35 clams per tank. Water was aerated continously and the experimental media was renewed every two days.

Clams were fed twice daily a culture of *Phaeodactylum tricornutum* corresponding to *c.* 3 mg dw . ind^{-1}. d^{-1} .

Sampling times were 0, 2, 5, 9, 14 and 20 days. At each time 6 to 8 clams, pooled from the two controls and the two copper containers, were taken for physiological measurements and 4 clams from each container were removed for copper analysis and determination of body condition index. This index is defined as the ratio dry weight : length of the shell (Bayne & Widdows, 1978).

The measurement of the physiological parameters were made as mentioned in Chapter 2. Feces for absorption efficiency measurements were collected from the containers within 5 to 6 hours of their production. Excretion rates were not measured as they constitute a negligible amount of lost energy (Widdows, 1993).

All the physiological measurements were standardized to 0.3 g dry weight, the average weight of the clams in the experiment.

All the procedures for copper analysis are described in Part I of this Chapter.

6.5.2.3. Results

No mortality occurred during the experiment.

Clearance rates are markedly lower in the copper exposed clams as it shown in Figure 6.8. This decline corresponds to 31.5 % (mean value) of the control. After an initial decline in clearance rate there was a tendency towards stabilization after the ninth day of exposure, at about 1.5 l. h^{-1} and 0.7 l. h^{-1}, in control and copper exposed clams respectively (i.e. 50 % of control).

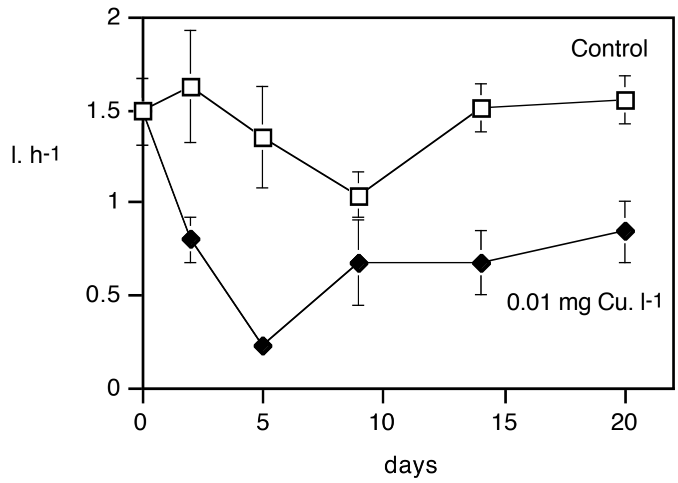

Fig. 6.8 - Variation of clearance rates (l. h^{-1} ± SE) in the control and copper exposed (0.01 mg Cu. l^{-1}) *Ruditapes decussatus* during the accumulation experiment.

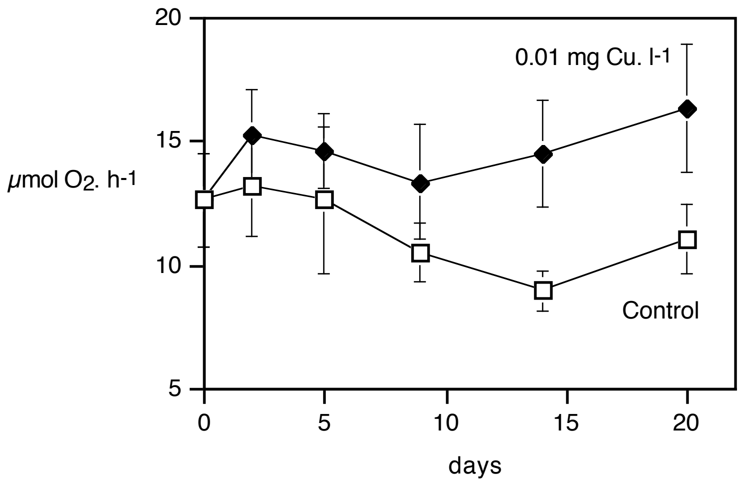

Fig.6.9 - Variation of respiration rates (µmol O_2. h^{-1} ± SE) in the control and copper exposed (0.01 mg Cu .l^{-1}) *Ruditapes decussatus* during the accumulation experiment.

In contrast, respiration rates were higher in the copper exposed clams (Figure 6.9). This enhancement is apparent within the first 2 days of exposure (120 %) and increases to *c.* 150 % by days 14 and 20.

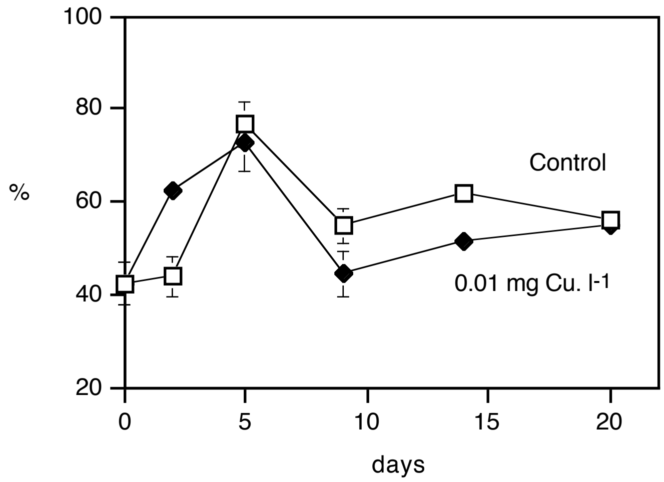

Fig. 6.10 - Variation of absorption efficiencies (% ± SE) in the control and copper exposed (0.01 mg Cu. l^{-1}) *Ruditapes decussatus* during the accumulation experiment.

Absorption efficiency follows the same pattern in both control and copper exposed clams, though values are generally lower for copper exposed clams (Figure 6.10). In the initial period (5 days) absorption efficiencies seem to compensate for the lower clearance rates, less food ingested but absorbed more efficiently. After the ninth day, there seems to be a tendency to stabilization slightly above 50 %. The amount of food given per day, though lower than environmental levels did not appear to influence body condition (Figure 6.11). Except for day 20 there are no significant differences in body condition of clams in the control and those exposed to copper. This maybe be due to continuing shell growth in the control, relative to dry weight increase.

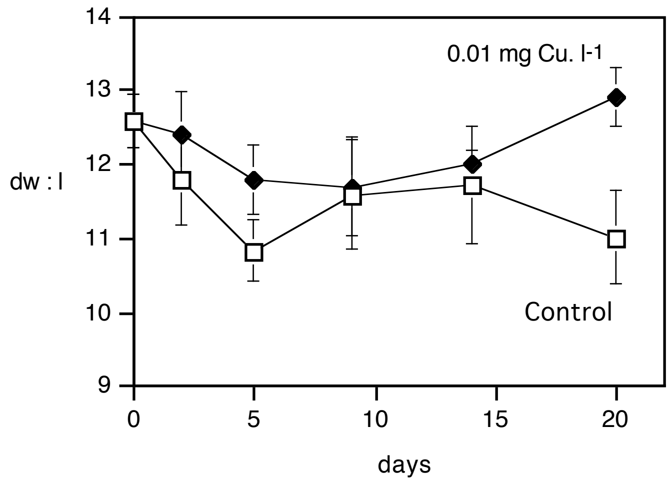

Fig. 6.11 - Variation of body condition index (ratio dry weight of soft parts:length of shell ± SE) of control and copper exposed *Ruditapes decussatus*, during the accumulation experiment.

Initial copper levels in the clams (4 µg Cu. g^{-1}), are lower than those normally found in the field (*c.* 7 - 11 µg Cu. g^{-1}, see Part I), probably resulting from depuration under laboratory conditions.

Copper is accumulated in the copper exposed clams throughout the 20 days exposure period (Figure 6.12). The rate of accumulation is higher during the first 48 hours, 1.95 d^{-1} , declining to 1.54 µg Cu. g^{-1} d^{-1} afterwards. Accumulation in the control, though much slower (0.59 µg Cu. g^{-1} d^{-1}), show evidence of an existing background contamination. Both in the control and in the copper exposed clams accumulation is linear and is explained by the regression equations in Figure 6.13. At the end of the experiment tissue copper concentrations of control clams were 15.1 µg Cu. g-1 dw, only slightly higher than field values. Copper exposed clams had a concentration of 31.8 µg Cu. g-1 dw and the bioconcentration factor, defined as the ratio copper in the tissues : copper in the water, was 3840.

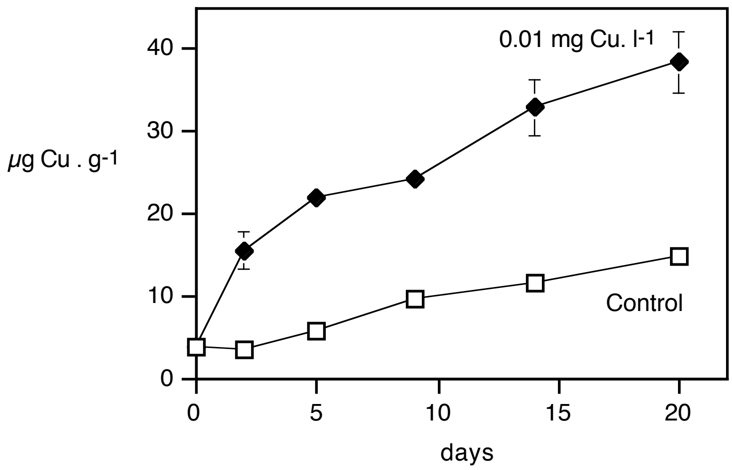

Fig. 6.12 - Variation of copper concentrations (µg. g $^{-1}$ dw) in the control and copper exposed (0.01 mg Cu. l^{-1}) *Ruditapes decussatus* during the accumulation experiment.

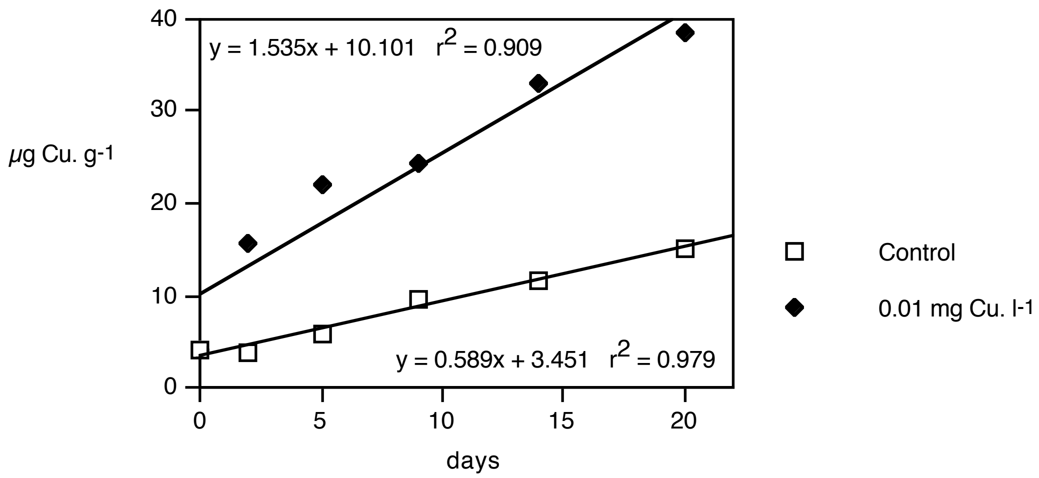

Fig. 6.13 - Regression lines for copper concentrations (µg. g^{-1} dw) in the control and copper exposed (0.01 mg Cu. l^{-1}) *Ruditapes decussatus* during the accumulation experiment (n = 6).

The physiological energetics and the main components of the energy budget in the control and the copper exposed clams are presented in

Figures 6.14 and 6.15. Scope for growth (SFG) was calculated for an average algal ration of 1.5 mg. l^{-1}. The shaded area, representing SFG, is always higher in the control than in the copper exposed clams.

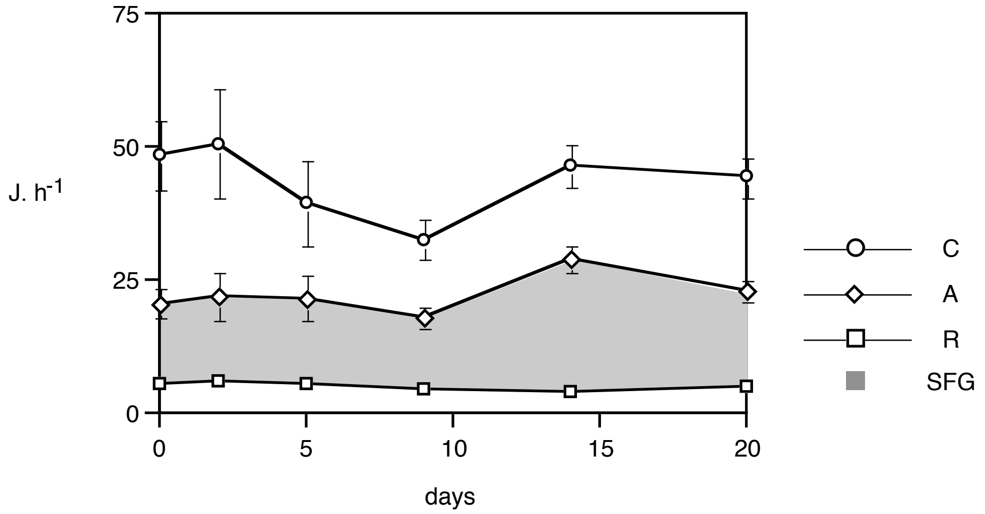

Fig. 6.14 - Energy budgets (J. h^{-1}) of control *Ruditapes decussatus* (0.3 g dw) during the accumulation experiment. C - energy consumed, A - energy absorbed, R - energy respired, shaded area represents scope for growth.

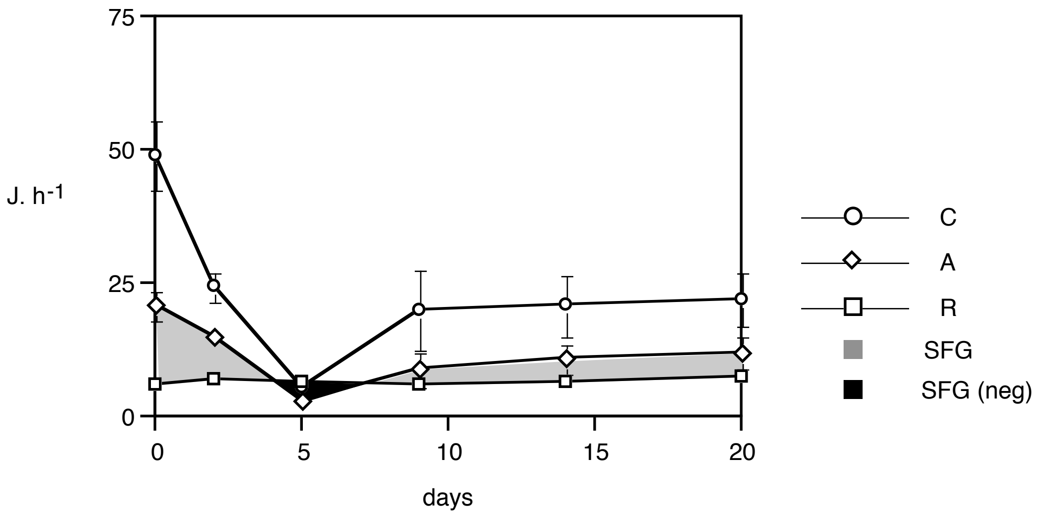

Fig. 6.15 - Energy budgets (J. h^{-1}) of copper exposed *Ruditapes decussatus* (0.3 g dw) during the accumulation experiment. C - energy consumed, A - energy absorbed, R - energy respired, shaded area represents scope for growth, black area corresponds to negative scope for growth.

Table 6.1. Components of energy budget (J. h^{-1}) for standard *Ruditapes decussatus* (0.3 g dw) during the accumulation experiment. C - energy consumed, AE - absorption efficiency, A - energy gain, R - energy loss through respiration, SFG - scope for growth.

Days	Copper concentration	**C** J. h^{-1}	**AE** %	**A** J. h^{-1}	**R** J. h^{-1}	**SFG** J. h^{-1}
0	Control	48.7	42.5	20.7	5.8	16.1
	0.01 mg. l^{-1}	48.7	42.5	13.920.7	5.8	16.1
2	Control	50.8	43.8	22.3	5.9	16.3
	0.01 mg. l^{-1}	24.2	62.6	15.2	6.9	8.4
5	Control	39.6	55.4	21.8	5.8	16.0
	0.01 mg. l^{-1}	5.6	55.4	3.1	6.7	-3.2
9	Control	32.8	54.8	17.9	4.8	12.5
	0.01 mg. l^{-1}	19.9	44.3	8.8	6.1	2.8
14	Control	46.8	62.1	29.0	4.1	23.9
	0.01 mg. l^{-1}	20.7	10.7	18.7	6.6	4.9
20	Control	47.8	55.9	26.8	5.1	23.1
	0.01 mg. l^{-1}	21.9	11.9	12.8	7.6	5.4

Even at the low copper concentration used in the experiment (0.01 mg Cu. l^{-1}), toxic effects can be detected at the physiological level. In this case, not only is scope for growth of copper exposed clams lower, but also it has a slightly negative value on the fifth day, indicating that the animals were having to utilize their body reserves to meet metabolic requirements. The components of the energy budget are given in Table 6.1.

SFG values of clams after 2 days exposure to 0.01 mg Cu. l^{-1} were not significantly different from SFG from the control, but by day 5 they were significantly lower (one-way ANOVA, $P < 0.01$). At the end of the experiment (14 - 20 days) differences became more significant (one-way ANOVA, $P < 0.001$).

No significant correlations were found between physiological responses and copper concentration in clams.

6.5.2.4. Discussion

The applicability of physiological energetics and scope for growth for stress assessment at environmental realistic copper concentrations is confirmed by the results obtained in this study. Scope for growth in the copper exposed clams was 23.5 % of the control scope for growth. These findings highlight the sensitivity of the physiological methodology and the integrated scope for growth measurement to environmentally relevant copper concentrations.

As expected at sub-lethal concentrations, no mortality occurred in the experiment and copper does not induce lethal effects during the exposure time considered. Using the same species, Stephenson & Taylor (1975), found no mortality until the 30th day in a sub-lethal assay at 15 °C, with the same copper concentration.

The copper accumulation shows two phases. An initial phase, from day 0 to day 5, when copper is rapidly accumulated, clearance rates decline markedly (lowest value 13.5 % of control) and respiration rates increased (116 % of control), resulting in very low values of scope for growth. The second phase (from day 9 on) when responses tend to stabilize indicating that the clams maybe limiting further toxic effects in spite of further copper accumulation.

It is known that many bivalves are able to avoid the toxic effects of several metals by complexing and sequestering them, particularly by producing metal binding proteins, such as metallothioneins (Viarengo *et al.*, 1980, 1981, Roesjadi, 1981). Due to their higher affinities for the most toxic cations, Hg, Cu, Cd (Viarengo & Canesi, 1991), they protect the cell structures from non-specific interaction of heavy metal cations with biological molecules and at the same time they can detoxify the excess of metal that penetrates into the cell (Viarengo *et al.*, 1987).

In mussels exposed to heavy metals the metallotheionein level greatly increases (Viarengo *et al.*, 1980, Roesjadi, 1982, Bebianno & Langston, 1991, 1992). Recent work with *R. decussatus* by Bebianno *et al.* (1993, 1994) show the metallothionein level is increased after exposure to sub-lethal concentrations of cadmium, especially in the gills, although it binds a smaller proportion of Cd than what was found for mussels.

This homeostasis mechanism thus can allow *R. decussatus* to regulate the toxicity of free metal copper cations in the cytosol, which would correspond to the “more stable” period of the accumulation curve.

The decrease in the physiological energetics in the first days can also be enhanced by the transient stress of adaptation to the experimental conditions, and thus can be misleading concerning the real toxic effects of copper. Bayne (1973, 1975) found a similar situation of transient stress when exposing mussels to low salinity stress, and verified that after 2 to 3 days their scope for growth recovered to normal values. In the case of salinity stress a complete compensation occurred while in the copper experiment only a partial compensation can be expected due to the deleterious effects of copper toxicity notably in the ciliary feeding activity. This is in fact shown by the decreased scope for growth values (negative on day 5) found in this initial period and that are always lower than those of the control.

The very low and even negative scope for growth found in the copper exposed clams show that in this initial period energy losses are higher than energy gain, probably due to the reduced feeding rate and the increased metabolic rate possibly reflecting the triggering of energy demanding detoxification metabolic processes, such as metal binding protein synthesis. In fact, it is in this early stage that copper shows a higher accumulation rate (1.95 μg Cu. g^{-1} dw d^{-1} in the first 48 hours).

In the second stage of the experiment, all values show a tendency to become more stable. The rate of copper uptake has lowered to 0.55 μg Cu. g^{-1} d^{-1} but scope for growth values are low (c. 23 % of the control values), clearance rate and energy gain is still reduced and the metabolic energy loss is enhanced. Scope for growth and performance of the clams is therefore still greatly affected, indicating that though animals partially recovered through detoxifying mechanisms, excess copper has caused sustained impairment of physiological functions. In fact, copper concentration having risen to 31.8 μg Cu. g^{-1} dw, clearance rates are 50%

of control rates,respiration rates are high, 145 % of control rates and SFG is reduced to *c.* 30 % of the initial value.

Very few contaminant accumulation studies have been performed with *R. decussatus*. In a previous study Sobral *et al.* (1992) and Sobral (1993) have found similar accumulation rates for the same copper concentration, 1.23 and 1.54 μg Cu. g^{-1} dw for 30 and 20 days experiment, respectively. Henry *et al.* (1984), Vicente *et al.* (1988) and Bebianno *et al* (1993, 1994) have studied cadmium accumulation but there are no studies relating the accumulation of metals to the physiological responses of this species.

Scope for growth is clearly dependent on factors such as food ration, and to a less extent, season and temperature, due to acclimation and the generally coupled response of clearance rates and respiration rates of bivalves. Hence in order to compare scope for growth values it is necessary to standardize conditions such as food ration, body size and season.

Comparisons with other works are therefore difficult, and also because methodologies are variable concerning the design of the experiment, toxicant concentrations, time of renewal, food levels, temperature, etc. The control scope for growth values of this study, when recalculated to other standard weights, are generally higher than values from experiments with *M. edulis* (Martin *et al.*, 1984, Sanders *et al.*, 1991, Widdows & Page, 1993), *R. phillipinarum* (Goulletquer *et al.*, 1989) using lower food levels.

Copper levels in the overlying water at the field site are highest (*c.* 5 μg. l^{-1}) in autumn/winter (see this Chapter, Part I). The interpolation between the SFG values found at the end of the accumulation experiment in the control and in the copper exposed clams, indicate that during those months clams may show a small reduction in SFG (11 %).

Though correlations have been found for mussels between SFG and toxicant concentration in the tissues (Gilfillan, 1980, Martin *et al.*, 1984, Widdows *et al.* 1987, 1990), SFG, clearance rates and body condition index (Martin *et al.*, 1984), we only found an expected significant correlation between scope for growth and food ration in the control, where toxicity was not enough to disrupt normal performance.

The initial decline in performance, which is responsible for the non significance of correlations, reflects the acute conditions of a short-term static exposure in contrast with the near steady-state conditions of the later phase of the experiment. According to Widdows & Page (1993), relating physiological effects (or toxicity) to toxicant concentrations is not appropriate in short-term static exposures when the water/tissue concentrations are far from equilibrium. This may be an explanation for the lack of correlations found in the present study and shows the advantage of utilizing flow-through systems when studying relationships between toxicant concentrations and physiological responses, because steady-state water/tissue concentrations can be achieved.

7. Conclusions

7. Conclusions

7.1. Physiological responses to hypoxia and anoxia

Clearance and respiration rates of *R. decussatus* do not show a parallel or coupled response to declining oxygen tensions. Clearance rate (or ventilation rate) can be maintained down to low oxygen levels compared to respiration rate which is greatly reduced below 50 % of air saturation.

R. decussatus, is able to maintain respiration rates independent of declining oxygen tensions down to 50 % of air saturation.

Respiration rates of *R. decussatus* exposed to oxygen tensions below 30 % of air saturation are maintained low and constant thus enabling the clam to still meet maintenance costs by aerobic catabolism.

Low anoxic metabolic rate and reduced energy expenditure enables *R. decussatus* to tolerate and survive periods of hypoxia which are likely to occur in its typical habitat.

Scope for growth is greatly diminished under extreme hypoxia, but it is not negative, indicating that even under low oxygen tensions, clams are not having to utilize their energy reserves.

7.2. Physiological responses to elevated temperature

Scope for growth of *R. decussatus* declines with raising temperatures indicating physiological stress for the clams in the temperature range 20-32 °C. High temperatures (above 27 °C) are stressful to the clams as shown by the low and even negative values of SFG.

Adaptation to tidal and seasonal temperature fluctuations causes the respiration rate of *R. decussatus* to be temperature independent within the range studied.

High temperatures (i.e. above 28 °C) and gaping during air exposure can significantly weaken clams resistance and affect their performance in water leading ultimately to death.

7.3. Physiological responses to current velocity, turbidity and particle size selection

Clearance rate of *R. decussatus* provides a good measure of impact and is also an index of the animal's ability to maintain its feeding activity and physiological condition when exposed to currents.

Increasing current velocity leads to decreasing clearance rates and ultimately to the inhibition of feeding in *R. decussatus*.

The slower currents support the highest clearance rates which is in agreement with field observations of clams living in enclosed sheltered areas, on the smooth slope banks of the intertidal.

The clams were able to cope with the mobile sediment maintaining some pumping activity to meet the oxygen requirements. The reduction of the siphons opening is the immediate response to the physical stimuli of sediment movement, thus causing clearance rate to decline and even cease by periodic valve closure.

The ability of jetting out depleted water at a different level than the one of the inhalant current enables *R. decussatus* to better exploit their environment, ensuring that the water is pumped in without particle dilution caused by the depleted exhalant current. This is an important adaptation by clams living in the slow currents of sheltered environments.

R. decussatus appears to control ingestion at high seston concentrations by reducing the amount filtered, lowering clearance rates and rejecting particles by producing pseudofeces.

Low clearance rates observed in response to high seston levels and mucus production are detrimental to performance.

Short settlement times after disturbance seem to reduce the clearance rate of *R. decussatus*, by *c.* 25 % at low seston concentrations.

R. decussatus does not efficiently retain particles smaller than 3 µm in diameter. Algal cells and other particles in the range 3 to 8 µm are efficiently retained, this being particularly beneficial when algal cells are diluted by the fine inorganic silt particles, as it is likely to occur in the clams natural environment.

7.4. Physiological responses to copper contamination

R. decussatus in the short term escapes the exposure to copper in seawater by valve closure and therefore acute tests are not applicable to adult clams of this species. Exposure times much longer than those generally accepted for acute tests are needed to establish the median lethal copper concentration (LC_{50}) for this species.

At environmental levels chronic exposure to copper does not induce lethal effects during the exposure time considered, but scope for growth is reduced by *c.* 25 %, indicating sustained impairment of physiological functions.

Copper is rapidly accumulated in the first days of exposure and physiological responses resulted in very low and negative values of scope for growth. After this initial period physiological responses tend to stabilize indicating that the clams maybe limiting further toxic effects (through homeostasis mechanisms) in spite of further copper accumulation.

No significant correlations where found between copper concentrations in the tissues and SFG, due to the initial decline in performance, which reflects the acute conditions of a short-term static exposure in contrast with the near steady-state conditions of the later phase of the experiment.

All the stress factors examined individually are detrimental of the physiological performance of *R. decussatus* with the consequent reduction in scope for growth. The applicability of physiological energetics and scope for growth for stress assessment is thus confirmed by the results obtained in this study.

Possible synergy between each of the examined stress factors, and with others not investigated in this study, such as parasitism, may further compromise the ecological fitness of the clams, but to what extent remains unknown.

These findings highlight the sensitivity of the physiological methodology and the integrated scope for growth measurement, not only as an early warning to irreversible environmental changes, when investigating the effects of contaminants, such as copper, but also to assess the effects of natural stress factors (such as hypoxia, elevated temperature, current velocity and turbidity) and the ability to compensate and adapt to a changing environment.

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8. References

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APPENDIX I

COMPOSITION AND PREPARATION OF THE CULTURE MEDIUM USED TO GROW *Phaeodactylum tricornutum*

	Add per 1 l of seawater
$NaNO_3$	75.0 mg
$NaH_2PO_4 . 2H_2O$	56.5 mg
Trace elements (stock solution 1)	1 ml
Vitamin mix (stock solution 2)	1 ml
Sodium metasilicate (stock solution 3)*	0.3 ml

Autoclave at 120 °C for 20 min at 1 atm

STOCK SOLUTIONS

1 - Trace elements:	Amounts per 1 l
$FeCl_3 . 6H_2O$	3.15 g
$CuSO_4 . 5H_2O$	0.010 g
$ZnSO_4 . 7H_2O$	0.022 g
$CoCl_2 . 6H_2O$	0.010 g
$MnCl_2 . 4H_2O$	0.180 g
$Na_2MO4 . 2H_2O$	0.006 g

2 - Vitamin mix:	Amounts per 1 l
Cyanocobalamin (B_{12})	0.5 mg
Thiamine HCl (B_1)	0.1 g

3 - Sodium metasilicate:	Amounts per 1 l
$Na_2SiO_3 . 5H_2O$	100.0 g

* Before adding stock solution 3, pH should be adjusted to 3.0-4.0 After addition, pH should be readjusted to 8.0.

APPENDIX II

FILTRATION AND GRAVIMETRIC ANALYSIS OF SUSPENDED PARTICLES

1. PREPARATION OF FILTER PAPERS FOR SESTON DETERMINATIONS

Soak filters in distilled water and wash excess fibres off. Replace water until it is clear (at least 3 times).

Remove excess water from filters using a vacuum pump

Dry overnight at 90 °C (on folded aluminium foil)

Number filters using a sharp pencil (so that the number will be marked and the filter not damaged.

Muffle at 450 °C for 4h (on folded aluminium foil and covered). Cool in desiccator (overnight) with fresh silica gel.

Weigh filters with fresh desiccant in the balance weighing chamber.

2. SESTON FILTRATION

Use 3 to 5 replicates for samples and blanks.

Throughly mix sample and immediately pour into a measuring cylinder. Gradually add sample to filter placed on filter holder and clamp funnel, ensuring that water in the cylinder is throughly mixed. Record the volume filtered. Care should be taken not to overload the filter.

Wash salts out using 3 times 5 ml of distilled water. Remove funnel and wash around filter edge (this is best done using a dripping bottle).

Fold filter carefully and dry at 90 °C until constant weight (usually 24 h). Cool in desiccator and weigh.

Blanks:

Wash 25 ml distilled water through filters, dry and weigh using to same procedures.

APPENDIX IV

PROCEDURES FOR THE PREPARATION OF SEDIMENT SAMPLES FOR DETERMINATION OF COPPER

In order to minimize contamination all materials that came into contact with the sediment were preferably made of high-density polyethylene (HDPE) and Teflon (TFE). These and the utilized labware were previously decontaminated overnight in a 1:1 solution of nitric acid (HNO_3). The agate mortars and balls were decontaminated for 10 min in a similar solution. The sieve was washed briefly with a 1:10 HNO_3 solution.

All the reagents used were of analytical grade and conductivity of distilled water (DW) was below 5 μS. cm^{-1}.

1. SAMPLE COLLECTION

Sediment samples were collected in HDPE boxes, kept cool and frozen upon arrival to the laboratory.

2. SAMPLE PREPARATION AND DIGESTION

Sediment was wet sieved (63 μm) to obtain the fine fraction in which copper concentration was to be determined. This fraction was oven dried at 60 °C for 48 hours and grinded to fine powder in a agate ball mill.

Samples of *c*. 0.7 g were wet digested in TFE digestion bombs with 3 ml of *aqua regia* (HNO_3 and chloridric acid HCl, 1:4) and 15 ml of fluoridric acid (HF). Digestion was performed in a covered water-bath at 100 °C during 1h 30 min.

The sample solutions were recuperated in 50 ml volumetric flasks to which a solution of 2.83 g of boric acid (HBO_3) in *c*. 5 ml DW had been previously added.

Prepared samples (50 ml) were kept cool (4 °C) until copper analysis were performed.

APPENDIX V

PROCEDURES FOR THE PREPARATION OF PARTICULATE SAMPLES FOR DETERMINATION OF COPPER

In order to minimize contamination all materials that came into contact with the sample were preferably made of high-density polyethylene (HDPE) and Teflon (TFE). Filter funnels were made of polypropylene (PP). These and the utilized labware were previously decontaminated overnight in a 1:1 solution of nitric acid (HNO_3). Filters were decontaminated for 1 h in a similar solution.

All the reagents used were of analytical grade and conductivity of distilled water (DW) was below 5 $\mu S. cm^{-1}$.

1. SAMPLE FILTRATION

Overlying seawater and pore water samples were collected in HDPE flasks and kept cool. Pore water samples were sieved *in situ* through a 200 µm sieve.

Water samples of known volume were filtered through polycarbonate Nuclepore® filters (0.4 µm pore size) to obtain the particulate matter. Filters, placed individually in plastic Petri dishes, were oven dried at 60 °C for 48 hours.

2. SAMPLE DIGESTION

Particulate matter samples were placed with plastic tweezers in TFE digestion bombs and wet digested with 1 ml of *aqua regia* (HNO_3 and chloridric acid HCl, 1:4) and 1 ml of fluoridric acid (HF). Digestion was performed in a covered water-bath at 100 °C during 1h. Filters are not dissolved by this procedure.

The sample solutions were recuperated in 25 ml volumetric flasks to which a solution of 0.93 g of boric acid (HBO_3) in *c.* 5 ml DW had been previously added.

Prepared samples (25 ml) were kept cool (4 °C) until copper analysis were performed.

APPENDIX VI

PROCEDURES FOR THE PREPARATION OF CLAM TISSUE SAMPLES FOR DETERMINATION OF COPPER

In order to minimize contamination all materials that came into contact with the soft parts of the clams were preferably made of high-density polyethylene (HDPE) and Teflon (TFE). These and the utilized labware were previously decontaminated overnight in a 1:1 solution of nitric acid (HNO_3). The agate mortars and balls were decontaminated for 10 min in a similar solution.

All the reagents used were of analytical grade and conductivity of distilled water (DW) was below 5 μS. cm^{-1}.

1. SAMPLE COLLECTION

Clams were unburied with a large blade knife and washed with seawater to remove excess sediment. They were kept cool in plastic net bags and returned to aerated seawater upon arrival to the laboratory. After a 48 hours period they were frozen (minimum 3-4 hours) and allowed to open. The soft parts were then washed with 0.5 M ammonium formate to remove salt and excised with a plastic knife. Clams individually placed in plastic layered Petri dishes were oven dried at 60 °C for 48 hours

2. SAMPLE DIGESTION

Dried clams were grinded to fine powder in a agate ball mill and *c.* 1 g sample was wet digested in a TFE digestion bomb with 5 ml of HNO_3 in a water-bath at 100 °C for 4 hours. 1 ml of hydrogen peroxide 30% (H_2O_2), was then added and digestion continued at the same temperature for another hour.

The sample solution was recuperated in 25 ml volumetric flasks with DW.

Prepared samples (25 ml) were kept cool (4 °C) until copper analysis were performed.